

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number  
**WO 01/19992 A2**

(51) International Patent Classification<sup>7</sup>: **C12N 15/13**,  
C07K 16/40, C12N 5/20, A61K 39/395, A61P 7/04

(21) International Application Number: PCT/EP00/08936

(22) International Filing Date:  
13 September 2000 (13.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
A 1576/99 14 September 1999 (14.09.1999) AT

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(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ,  
BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK,  
DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,  
IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU,  
LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT,  
RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA,  
UG, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,  
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— Without international search report and to be republished  
upon receipt of that report.

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: **FACTOR IX/FACTOR IXa ANTIBODIES AND ANTIBODY DERIVATIVES**

(57) Abstract: An antibody or antibody derivative against factor IX/activated factor IX (FIXa) which increases the procoagulant activity of FIXa.

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The present invention relates to factor IX/factor IXa-antibodies and antibody derivatives.

5        Blood clots (thrombi) are formed by a series of  
zymogen activations referred to as the coagulation  
cascade. In the course of this enzymatic cascade, the  
activated form of each of such zymogens (referred to as  
factors) catalyzes the activation of the next one.  
10    Thrombi are deposits of blood components on the surface  
of a blood vessel wall and mainly consist of aggregated  
blood platelets and insoluble, cross-linked fibrin.  
Fibrin formation is effected by means of thrombin by  
limited proteolysis of fibrinogen. Thrombin is the final  
15    product of the coagulation cascade, (K.G. Mann, Blood,  
1990, Vol. 76, pp.1-16).

Activation of factor X by the complex of activated  
factor IX (FIXa) and activated factor VIII (FVIIIa) is a  
key step in coagulation. The absence of the components  
20    of this complex or a disturbance of their function is  
associated with the blood coagulation disorder called  
hemophilia (J.E. Sadler & E.W. Davie: Hemophilia A,  
Hemophilia B and von Willebrand's disease, in G.  
Stamatoyannopoulos et al. (Eds.): The molecular basis of  
25    blood diseases. W.B. Saunders Co., Philadelphia, 1987,  
pp. 576-602). Hemophilia A denotes a (functional)  
absence of factor VIII activity, while Hemophilia B is  
characterized by the absence of factor IX activity.  
At present, treatment of Hemophilia A is effected via a  
30    substitution therapy by administering factor VIII  
concentrates. However, approximately 20-30% of  
Hemophilia A patients develop factor VIII inhibitors  
(i.e. antibodies against factor VIII), whereby the

effect of administered factor VIII preparations is inhibited. Treatment of factor VIII inhibitor patients is very difficult and involves risks, and so far there exist only a limited number of treatments for these patients.

In the case of patients having a low FVIII inhibitor level, it is possible, though expensive, to administer high doses of factor VIII to such patients and thus to neutralize the antibodies against factor VIII. The amount of factor VIII beyond that needed to neutralize the inhibitor antibodies then has hemostatic action. In many cases, desensitization can be effected, whereupon it is then possible again to apply standard factor VIII treatments. Such high dose factor VIII treatments require, however, large amounts of factor VIII, are time-consuming and may involve severe anaphylactic side reactions. Alternatively, the treatment may be carried out with porcine factor VIII molecules.

A further high-cost method involves removing factor VIII inhibitors through extra corporeal immunoadsorption on lectins which bind to immunoglobulins (protein A, protein G) or to immobilized factor VIII. Since the patient must be connected to an apheresis machine during this treatment, the treatment also constitutes a great burden on the patient. It is also not possible to treat an acute hemorrhage in this way.

At present, the therapy of choice is to administer activated prothrombin complex concentrates (APCC), such as FEIBA® and AUTOPLEX®, which are suitable for the treatment of acute hemorrhages even in patients having a high inhibitor titer (DE 31 27 318).

In the intravascular system of blood coagulation, the last step is the activation of factor X. This reaction is stimulated by the binding of factor VIIIIa to factor IXa and the formation of a "tenase"-complex  
5 consisting of the factors IXa, VIIIIa, X and phospholipid. Without the binding of FVIIIIa, FIXa exhibits no or only a very slight enzymatic activity relative to FX.

Over the last several years, a number of possible  
10 binding sites for factor VIIIIa to factor IXa have been characterized, and it has been shown that antibodies or peptides which bind to these regions inhibit the activity of FIXa (Fay et al., J. Biol. Chem., 1994, Vol.269, pp.20522-20527, Lenting et al., J. Biol. Chem.,  
15 1996, Vol. 271, pp. 1935-1940, Jorquera et al., Circulation, 1992, Vol. 86, Abstract 2725). The inhibition of coagulation factors, such as factor IX, has also been achieved through the use of monoclonal antibodies with the aim of preventing thrombosis  
20 formation (WO 97/26010).

The opposite effect, i.e. an increase in the factor IXa mediated activation of factor X, has been described by Liles D.K. et al., (Blood, 1997, Vol. 90, suppl. 1, 2054) through the binding of a factor VIII peptide  
25 (amino acids 698-712) to factor IX. Yet, this effect only occurs in the absence of factor VIIIIa, while in the presence of factor VIIIIa the factor IXa/factor VIIIIa-mediated cleavage of factor X is inhibited by this peptide.

30

#### SUMMARY OF THE INVENTION

With a view to the possible risks and side effects which may occur in the treatment of hemophilia patients,

there is a need for a therapy which allows for the effective treatment of FVIII inhibitor patients.

Therefore, it is an object of the present invention to provide a preparation for the treatment of blood  
5 coagulation disorders which has particular advantages for factor VIII inhibitor patients.

According to the present invention, this object is achieved through the use of antibodies or antibody derivatives against factor IX/factor IXa which have  
10 factor VIIId-cofactor activity or factor IXa-activating activity and lead to an increase in the procoagulant activity of factor IXa. Surprisingly, the action of these inventive factor IX/factor IXa-activating antibodies and antibody derivatives is not negatively  
15 affected by the presence of inhibitors, such as inhibitors against factor VIII/factor VIIId, but instead the procoagulant activity of factor IXa in this case also is increased.

A further advantage of this invention is that the  
20 administration of the preparation according to the invention allows for rapid blood coagulation even in the absence of factor VIII or factor VIIId, even in the case of FVIII inhibitor patients. Surprisingly, these agents are also effective in the presence of factor VIIId.

25 The antibodies and antibody derivatives according to the present invention thus have a FVIII-cofactor-like activity which, in a FVIII assay (e.g. a COATEST® assay or Immunochrom test) after 2 hours of incubation exhibits a ratio of background (basic noise) to measured  
30 value of at least 3. Calculation of this ratio may, e.g., be effected according to the following scheme:

Antibody measurement (OD 405) – blank value from reagent > 3  
Mouse-IgG-measurement (OD 405) – blank value from reagent  
after two hours of incubation.

5       The antibodies according to the invention preferably have an in vivo half life of at least 5 days, more preferably at least 10 days, though it is more preferred to have a half life of at least 20 days.

10       A further aspect of this invention is a preparation comprising antibodies and/or antibody derivatives against factor IX/factor IXa and a pharmaceutically acceptable carrier substance. Furthermore, the preparation according to the invention may additionally comprise factor IX and/or factor IXa.

15       A further aspect of the invention is the use of the antibodies or antibody derivatives to increase the amidolytic activity of factor IXa.

20       Fig. 1 shows the results of a screening of supernatants from hybridoma cell cultures for FVIII-like activity. Pre-selected clones from fusion experiments, #193, #195 and #196, were tested in a chromogenic assay.

      Fig. 2 shows the results of screening for IgG-mediated factor VIII-like activity in supernatants of a hybridoma cell culture of a master plate.

25       Fig. 3 shows the subcloning of clone 193/C0, namely the results of the first cloning round.

      Fig. 4 shows a comparison of the chromogenic FVIII-like activity and factor IX-ELISA-reactivity of hybridoma cultures derived from the starting clone  
30   193/C0.

      Fig. 5 shows the results of the measurement of the chromogenic activity of some master clones and sub-clones.

Fig. 6A shows the FVIII-like activity of the anti-FIX/FIXa-antibodies 193/AD3 and 196/AF2 compared to human FVIII, TBS buffer and cell culture medium. After a lag phase, both antibodies gave rise to chromogenic substrate cleavage, as judged by the increasing optical density.

Fig. 6B shows a comparison of the chromogenic activity of factor VIII, 196/AF1, 198/AC1/1 and mouse-IgG.

Fig. 7A shows a comparison of the kinetics of Factor Xa generation by Factor VIII and 196/AF2 with and without the addition of a Factor Xa specific inhibitor.

Fig. 7B shows a comparison of the kinetics of the Factor Xa generation by Factor VIII, mouse-IgG and anti-factor IX/IXa-antibody 198/AM1 with and without the addition of a factor Xa-specific inhibitor, Pefabloc Xa®.

Fig. 8A shows a measurement of the dependence of the factor VIII-like activity of purified anti-factor IX/IXa-antibody 198/AC1/1 in the presence and absence of phospholipids, FIXa/FX and calcium ions.

Fig. 8B shows a measurement of the dependence of FXa generation by anti-FIXa-antibody 196/AF1 in the presence of phospholipids,  $\text{Ca}^{2+}$  in FIXa/FX.

Fig. 8C shows the generation of FXa by unspecific mouse IgG antibody.

Fig. 9 is a graphical representation of the coagulation times of Factor VIII-deficient plasma in an APTT assay by using various concentrations of anti-factor IX/IXa-antibody 193/AD3.

Fig. 10A shows that in the presence of Factor IXa, antibody 193/AD3 leads to a reduction in the coagulation time of factor VIII-deficient plasma.

Fig. 10B shows a dose-dependent reduction of the clotting time by antibody 193/AD3 in the presence of factor IXa- and factor VIII-inhibitors.

Fig. 11 shows the chromogenic activity of  
5 antibodies 198/A1, 198/B1 and 198/AP1 in the presence and absence of human FIXa $\beta$ .

Fig. 12 shows the primer sequences for the amplification of the genes of the variable heavy chain of mouse antibody.

10 Fig. 13 shows the primer sequences for the amplification of the genes of the variable light (kappa) chain of the mouse antibody.

Fig. 14 shows the DNA and derived protein sequence of the scFv from hybridoma cell line 193/AD3  
15 (SEQ.ID.NOs. 81 and 82).

Fig. 15 shows the DNA and derived protein sequence of the scFv from hybridoma cell line 193/K2 (SEQ.ID.NOs. 83 and 84).

Fig. 16 shows the DNA and derived protein sequence  
20 of the scFv from hybridoma cell line 198/AB2 (subclone of 198/B1) (SEQ.ID.NOs. 85 and 86).

Fig. 17 shows the DNA and deduced protein sequence of scFv derived from the cell line 198/A1 (SEQ.ID.NOs. 87 and 88).

25 Fig. 18 demonstrates the chromogenic FVIII-like activity of peptide A1/3 in the presence of 2.9nM human FIXa. The scrambled version of peptide A1/3, peptide A1/5 does not give rise to any FXa generation.

Fig. 19 demonstrates the dependence of the  
30 chromogenic FVIII-like activity of peptide A1/3 on the presence of human FIXa. In the absence of human FIXa, peptide A1/3 does not give rise to any FXa generation. The buffer control, plain imidazole buffer is designated



IZ.

Fig. 20 shows that the chirality of Arg-residues does not play a significant role for the chromogenic activity of peptides A1/3-rd and A1/3-Rd-srmb.

5 Fig. 21 shows that the addition of 2.4 $\mu$ M peptide B1/7 to the reaction mixture led to a measureable generation of Fxa.

Fig. 22 shows that the addition of a FX-specific inhibitor results in a significant reduction in the reaction. If there was no FIXa and FX is added to the reaction mixture, no Fxa was synthesized.

Fig. 23 shows vector pBax-IgG1.

Fig. 24 shows the increase of the amidolytic activity of FIXa in the presence of antibody 198/B1 (Fig. 24A) and IgM antibody 198/AF1 (Fig. 24B).

15 Fig. 25 demonstrates the chromogenic FVIII-like activity of the antibody 198/A1 Fab fragment in the presence of 2.3nM human FIXa. As a positive control the intact antibody 198/A1 was used as well as 7.5pM FVIII. The buffer control (IZ) was used as a negative control.

Fig. 26 shows the nucleotide and amino acid sequence of the 198AB2 scFv-alkaline phosphatase fusion protein (ORF of the expression vector pDAP2-198AB2#100, (SEQ.ID.NOs. 89 and 90).

25 The genes for the VL and the VH domains of antibody 198/AB2 (198/AB2 is an identical subclone of 198/B1) were derived from the corresponding hybridoma cells as described in example 10. The PCR product of the VH-gene was digested SfiI - AscI and the PCR-product of the VL-gene was digested AscI and NotI. VH and VL genes were linked via the AscI site and inserted into SfiI - NotI digested vector pDAP2 (Kerschbaumer R.J. et al, Immunotechnology 2, 145-150, 1996; GeneBank accession

No.:U35316). PelB leader: leader sequence of *Erwinia carotovora* Pectate Lyase B, His tag, Histidine tag for metal ion chromatography.

Fig. 27 demonstrates the chromogenic FVIII-like activity of two antibody 198/B1 (subclone AB2) scFv fragment-alkaline phosphatase fusion proteins (198AB2#1 and 198AB2#100) in the presence of 2.3nM human FIXa. As a positive control 7.5pM FVIII was used.

Fig. 28 shows the amino acid and nucleotide sequence of pZip198AB2#102 (SEQ.ID.NOs. 91 and 92).

Fig. 29 shows the nucleotide and amino acid sequence of the mAB#8860 scFv-alkaline phosphatase fusion protein (vector pDAP2-8860scFv#11, (SEQ.ID.NOs. 93 and 94). The genes for the VL and the VH domains of antibody #8860 were derived from the corresponding hybridoma cells as described in example 10. The PCR product of the VH-gene was digested SfiI - AscI and the PCR-product of the VL-gene was digested AscI and NotI. VH and VL genes were linked via the AscI site and inserted into SfiI - NotI digested vector pDAP2 (Kerschbaumer R.J. et al, Immunotechnology 2, 145-150, 1996; GeneBank accession No.:U35316).

Fig. 30 shows the nucleotide and amino acid sequence of the mAB #8860 scFv-leucine zipper fusion protein (miniantibody; vector p8860-Zip#1.2, (SEQ.ID.NOs. 95 and 96). The gene of the scFv fragment was derived from mAB #8860 and was swapped from vector pDAP2-8860scFv#11 into SfiI-NotI digested plasmid pZip1 (Kerschbaumer R.J. et al., Analytical Biochemistry 249, 219-227, 1997; GeneBank accession No.: U94951)

Fig. 31 demonstrates the chromogenic FVIII-like activity of the 198/B1 (subclone AB2) miniantibody 198AB-Zip#102 in the presence of 2.3nM human FIXa. As a

positive control 4.8pM FVIII was used whereas a unrelated miniantibody (8860-Zip#1.2) and plain reaction buffer (IZ) served as negative controls.

Fig. 32 shows a schematic representation of the  
5 plasmid pMyHis6.

Fig. 33 shows the nucleotide and amino acid sequence of the part of the plasmid pMyHis6 differing from vector pCOCK (SEQ.ID.NOs. 97 and 98). Vector pMyHis6 was constructed by cleaving vector pCOCK  
10 (Engelhardt et al., 1994, Biotechniques, 17:44-46) with NotI and EcoRI and insertion of the oligonucleotides: mychis6-co: 5'ggccgcagaacaaaaactcatctcagaagaggatct gaatggggcgggcacatcaccatcaccatcactaataag 3' (SEQ ID.No. 79) and mycchis-ic:  
15 5'aattcttattagtgatggtgatggtgatgtgccgccccattcagatcctcttct gagatgagtttttgttctgc (SEQ.ID.No. 80).

Fig. 34 shows the nucleotide and amino acid sequence of 198AB2 scFv (linked to the c-myc-tag and the His6- tag): ORF of the expression vector pMyHis6-  
20 198AB2#102. Vector pMyHis6 was constructed by cleaving vector pCOCK (Engelhardt O. et al, BioTechniques 17, 44-46, 1994) NotI - EcoRI and inserting the following annealed oligonucleotides:

(5'-GGCCGCAGAACAAAACTCATCTCAGAAGAGGATCTGAATGGG  
25 GCGGCACATCACCATCACCATCACTAATAAG - 3' (SEQ.ID.No. 103)

and

5'- TTATTAGTGATGGTGATGGT  
GATGTGCCGCCCCATTGAGATCCTCTTCTGAGATGAGTTTTGTTCTGC-  
3' (SEQ.ID.NO. 104)). The resultant vector, named  
30 pMyHis6, was cleaved SfiI - NotI and the gene of scFv 198AB2 was swapped into this vector from vector pDAP2-198AB2#100.

Fig. 35 shows the nucleotide and amino acid

sequence of the mAB #8860 scFv linked to the c-myc-tag and the His6- tag (vector p8860-M/H#4c, SEQ.ID.NOs. 101 and 102). Plasmid pMyHis6 was cleaved with SfiI and NotI and the DNA sequence coding for the scFv 8860#11  
5 protein was inserted from pDAP2-8860scFv#11 (see Fig.29) yielding plasmid p8860-M/H#4c.

Fig. 36 demonstrates the chromogenic FVIII-like activity of the 198/B1 (subclone AB2) scFv fragment (MyHis-198AB2#102) in the presence of 2.3nM human FIXa.  
10 As a positive control 4.8pM FVIII was used whereas a unrelated scFv (8860-M/H#4c) and plain reaction buffer (IZ) served as negative controls.

#### **Antibodies and Antibody Derivatives.**

15 The present invention also comprises the nucleic acids encoding the inventive antibodies and antibody derivatives, expression vectors, hybridoma cell lines, and methods for producing the same.

Antibodies are immunoglobulin molecules having a  
20 specific amino acid sequence which only bind to antigens that induce their synthesis (or its immunogen, respectively) or to antigens (or immunogens) which are very similar to the former. Each immunoglobulin molecule consists of two types of polypeptide chains. Each  
25 molecule consists of large, identical heavy chains (H chains) and two light, also identical chains (L chains). The polypeptides are connected by disulfide bridges and non-covalent bonds. In vivo, the heavy and light chains are formed on different ribosomes, assembled in the  
30 cell, and secreted as intact immunoglobulins (Roitt I. et al., in: Immunology, second ed., 1989).

The inventive antibodies and antibody derivatives and organic compounds derived there from comprise human

and animal monoclonal antibodies or fragments thereof, single chain antibodies and fragments thereof and miniantibodies, bispecific antibodies, diabodies, triabodies, or di-, oligo- or multimers thereof. Also  
5 included are peptidomimetics or peptides derived from the antibodies according to the invention, e.g. they comprise one or several CDR regions, preferably the CDR3 region.

Further included are human monoclonal antibodies  
10 and peptide sequences which, based on a structure activity connection, are produced through an artificial modeling process (Greer J. et al., J. Med. Chem., 1994, Vol. 37, pp. 1035-1054).

The term factor IX/IXa activating antibodies and  
15 antibody derivatives may also include proteins produced by expression of an altered, immunoglobulin-encoding region in a host cell, e.g. "technically modified antibodies" such as synthetic antibodies, chimeric or humanized antibodies, or mixtures thereof, or antibody  
20 fragments which partially or completely lack the constant region, e.g. Fv, Fab, Fab' or F(ab)'<sub>2</sub> etc. In these technically modified antibodies, e.g., a part or parts of the light and/or heavy chain may be substituted. Such molecules may, e.g., comprise  
25 antibodies consisting of a humanized heavy chain and an unmodified light chain (or chimeric light chain), or vice versa. The terms Fv, Fc, Fd, Fab, Fab' or F(ab)<sub>2</sub> are used as described in the prior art (Harlow E. and Lane D., in "Antibodies, A Laboratory Manual", Cold  
30 Spring Harbor Laboratory, 1988).

The present invention also comprises the use of Fab fragments or F(ab)<sub>2</sub> fragments which are derived from monoclonal antibodies (mAb), which are directed against

factor IX/factor IXa and cause an increase of the procoagulant activity of factor IXa.

Preferably, the heterologous framework regions and constant regions are selected from the human

5 immunoglobulin classes and isotypes, such as IgG (subtypes 1 to 4), IgM, IgA and IgE. In the course of the immune response, a class switch of the immunoglobulins may occur, e.g. a switch from IgM to IgG; therein, the constant regions are exchanged, e.g. from  $\mu$   
10 to  $\gamma$ . A class switch may also be caused in a directed manner by means of genetic engineering methods ("directed class switch recombination"), as is known from the prior art (Esser C. and Radbruch A., Annu. Rev. Immunol., 1990, Vol. 8, pp. 717-735). However, the  
15 antibodies and antibody derivatives according to the present invention need not comprise exclusively human sequences of the immunoglobulin proteins.

In one particular embodiment, a humanized antibody comprises complement determining regions (CDRs) from  
20 murine monoclonal antibodies which are inserted in the framework regions of selected human antibody sequences. However, human CDR regions can also be used. Preferably, the variable regions in the human light and heavy chains are technically altered by one or more CDR exchanges. It  
25 is also possible to use all six CDRs or varying combinations of less than six CDRs.

The humanized antibody according to the present invention preferably has the structure of a human antibody or of a fragment thereof and comprises the  
30 combination of characteristics necessary for a therapeutic application, e.g., the treatment of coagulation disorders in patients, preferably factor VIII inhibitor patients.

A chimeric antibody differs from a humanized antibody in that it comprises the entire variable regions including the framework regions of the heavy and light chains of non-human origin in combination with the constant regions of both chains from human immunoglobulin. A chimeric antibody consisting of murine and human sequences may, for example, be produced. According to the present invention, the antibodies and antibody derivatives may also be single chain antibodies or miniantibodies (scFv fragments, which, e.g., are linked to proline-rich sequences and oligomerisation domains, e.g. Pluckthun A. and Pack P., Immunotechnology, 1997, Vol. 3, pp. 83-105) or single chain Fv (sFv) which incorporate the entire antibody binding region in one single polypeptide chain. For instance, single chain antibodies may be formed by linking the V-genes to an oligonucleotide which has been constructed as a linker sequence and connects the C terminus of the first V region with the N terminus of the second V region, e.g. in the arrangement VH-Linker-VL or VL-Linker-VH; both,  $V_H$  and  $V_L$  thus may represent the N-terminal domain (Huston JS et al., Int. Rev. Immunol., 1993, Vol. 10, pp. 195-217; Raag R. and Whitlow M., FASEB J., 1995, Vol. 9, pp. 73-80). The protein which can be used as linker sequence may, e.g., have a length of up to 150 Å, preferably up to 80 Å, and more preferably up to 40 Å. Linker sequences containing glycine and serine are particularly preferred for their flexibility, or glutamine and lysine, respectively, for their solubility. The choice of the amino acid is effected according to the criteria of immunogenicity and stability, also depending on whether or not these single chain antibodies are to be suitable for physiological or

industrial applications (e.g. immunoaffinity chromatography). The single chain antibodies may also be present as aggregates, e.g. as trimers, oligomers or multimers. The linker sequence may, however, also be missing, and  
5 the connection of the  $V_H$  and  $V_L$  chains may occur directly.

Bispecific antibodies are macromolecular, heterobifunctional cross-linkers having two different binding specificities within one single molecule. In  
10 this group belong, e.g., bispecific (bs) IgGs, bs IgM-IgAs, bs IgA-dimers, bs (Fab')<sub>2</sub>, bs(scFv)<sub>2</sub>, diabodies, and bs bis Fab Fc (Cao Y. and Suresh M.R., Bioconjugate Chem., 1998, Vol. 9, pp. 635-644).

By peptidomimetics, protein components of low  
15 molecular weight are understood which imitate the structure of a natural peptide component, or of templates which induce a specific structure formation in an adjacent peptide sequence (Kemp DS, Trends Biotechnol., 1990, pp. 249-255). The peptidomimetics  
20 may, e.g., be derived from the CDR3 domains. Methodical mutational analysis of a given peptide sequence, i.e. by alanine or glutamic acid scanning mutational analysis, allows for the identification of peptide residues critical for procoagulant activity. Another possibility  
25 to improve the activity of a certain peptide sequence is the use of peptide libraries combined with high throughput screening.

The term antibodies and antibody derivatives may also comprise agents which have been obtained by  
30 analysis of data relating to structure-activity relationships. These compounds may also be used as peptidomimetics (Grassy G. et al., Nature Biotechnol.,



1998, Vol. 16, pp. 748-752; Greer J. et al., J. Med. Chem., 1994, Vol. 37, pp. 1035-1054).

Examples of hybridoma cells expressing the antibodies or antibody derivatives according to the invention were deposited on 9 September 1999 under the numbers 99090924 (#198/A1), 99090925 (#198/B1) and 99090926 (#198/BB1) and on December 16, 1999 under the numbers 99121614 (#193/A0), 99121615 (#196/C4), 99121616 (#198/D1), 99121617 (198/T2), 99121618 (#198/G2), 99121619 (#198/AC1) and 99121620 (#198/U2) according to the Budapest Treaty.

#### **Methods of Production:**

The antibodies of the present invention can be prepared by methods known from the prior art, e.g. by conventional hybridoma techniques, or by means of phage display gene libraries, immunoglobulin chain shuffling or humanizing techniques (Harlow E. and Lane D., in: Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, 1988). The production of the inventive antibodies and antibody derivatives may, for instance, be made by conventional hybridoma techniques (Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, 1988, Eds. Harlow and Lane, pp. 148-242). According to the present invention, human and also non-human species may be employed therefor, such as cattle, pigs, monkeys, chickens and rodents (mice, rats). Normal, immunocompetent Balb/c mice or FIX-deficient mice may, e.g., be used (factor IX-deficient mice may be obtained from Dr. Darrel Stafford from the University of North Carolina, Chapel Hill). Immunization may, e.g., be effected with factor IX, factor IX $\alpha$  or completely activated factor IX $\alpha\beta$ , or with fragments thereof.

The hybridomas are selected with a view to the fact that the antibodies and antibody derivatives in the supernatants of the hybridoma cells bind to factor IX/factor IXa and cause an increase of the procoagulant activity of factor IXa. The increase in the procoagulant activity may, e.g., be proven by assaying methods as known from the prior art for the measurement of factor VIII-like activity, e.g. chromogenic assays.

Alternatively, the antibodies and antibody derivatives of the invention may also be produced by recombinant production methods. In doing so, the DNA sequence of the antibodies according to the invention can be determined by known techniques, and the entire antibody DNA or parts thereof can be expressed in suitable systems. Recombinant production methods can be used, such as those involving phage display, synthetic and natural libraries, expression of the antibody proteins in known expression systems, or expression in transgenic animals (Jones et al., Nature, 1986, Vol. 321, pp.522-525; Phage Display of Peptides and Proteins, A Laboratory Manual, 1996, Eds. Kay et al., pp. 127-139; US 4,873,316; Vaughan T.J. et al., Nature Biotechnology, 1998, pp. 535-539; Persic L. et al., Gene, 1997, pp. 9-18; Ames R.S. et al., J.Immunol.Methods, 1995, pp. 177-186).

The expression of recombinantly produced antibodies may be effected by means of conventional expression vectors, such as bacterial vectors, such as pBr322 and its derivatives, pSKF or eukaryotic vectors, such as pMSG and SV40 vectors. Those sequences which encode the antibody may be provided with regulatory sequences which regulate the replication, expression and secretion from

the host cell. These regulatory sequences comprise promoters, e.g. CMV or SV40, and signal sequences.

The expression vectors may also comprise selection and amplification markers, such as the dihydrofolate reductase gene (DHFR), hygromycin-B- phosphotransferase,  
5 thymidine-kinase etc.

The components of the vectors used, such as selection markers, replicons, enhancers etc., may either be commercially obtained or prepared by means of  
10 conventional methods. The vectors may be constructed for the expression in various cell cultures, e.g. for mammalian cells such as CHO, COS, fibroblasts, insect cells, yeast or bacteria, such as E. coli. Preferably, those cells are used which allow for an optimal  
15 glycosylation of the expressed protein. Particularly preferred is the vector pBax (cf. Fig. 17) which is expressed in CHO cells or in SK-Hep.

The production of Fab fragments or F(ab)<sub>2</sub> fragments may be effected according to methods known from the prior  
20 art, e.g. by cleaving a mAb with proteolytic enzymes, such as papain and/or pepsin, or by recombinant methods. These Fab and F(ab)<sub>2</sub> fragments may also be prepared by means of a phage display gene library (Winter et al., 1994, Ann. Rev. Immunol., 12:433-455).

25 The antibody derivatives may also be prepared by means of methods known from the prior art, e.g. by molecular modeling, e.g. from Grassy G. et al., Nature Biotechnol., 1998, Vol. 16, pp. 748-752, or Greer J. et al., J. Med. Chem., Vol. 37, pp. 1035-1054, or Rees A.  
30 et al., in: "Protein Structure Prediction: A practical approach", ed. Sternberg M.J.E., IRL press, 1996, chapt. 7-10, pp. 141-261.

The purification of the inventive antibodies and antibody derivatives may also be carried out by methods described in the prior art, e.g., by ammonium sulfate precipitation, affinity purification (protein G-  
5 Sepharose), ion exchange chromatography, or gel chromatography. The following methods may be used as the test methods to show that the antibodies and antibody derivatives of the present invention bind to factor IX/factor IXa, increase the procoagulant activity of  
10 factor IXa or have factor VIII-like activity.: the one step coagulation test (Mikaelsson and Oswaldson, Scand. J. Haematol., Suppl., 33, pp. 79-86, 1984) or the chromogenic tests, such as COATEST VIII:C® (Chromogenix) or Immunochrom (IMMUNO). In principle, all the methods  
15 used for determining factor VIII activity may be used. As the control blank value for the measurements, e.g., unspecific mouse-IgG antibody may be used.

The present antibodies and antibody derivatives are suitable for therapeutic use in the treatment of  
20 coagulation disorders, e.g. in the case of hemophilia A, for factor VIII inhibitor patients etc. Administration may be effected by any method suitable to effectively administer the therapeutic agent to the patient, e.g. by oral, subcutaneous, intramuscular, intravenous or  
25 intranasal administration.

Therapeutic agents according to the invention may be produced as preparations which comprise a sufficient amount of antibodies or of antibody derivatives as the active agent in a pharmaceutically acceptable carrier  
30 substance. These agents may be present either in liquid or in powderized form. Moreover, the preparations according to the invention may also comprise mixtures of different antibodies, the derivatives thereof and/or

organic compounds derived therefrom, as well as mixtures consisting of antibodies and factor IX and/or factor IXa. Factor IXa may be present as factor IXa $\alpha$  and/or factor IXa $\beta$ . An example of an aqueous carrier substance  
5 is, e.g., saline. The solutions are sterile, sterilisation being effected by conventional methods.

The antibodies or antibody derivatives according to the invention may be present in lyophilized form for storage and be suspended in a suitable solvent before  
10 administration. This method has proven generally advantageous for conventional immunoglobulins, and known lyophilisation and reconstitution methods may be applied in this case.

Moreover, the antibodies and antibody derivatives  
15 according to the invention may also be used for industrial applications, e.g. for the purification of factor IX/factor IXa by means of affinity chromatography, or as a component of detection methods (e.g. ELISA assays), or as an agent for identification of and  
20 interaction with functional domains of a target protein.

The present invention will be described in more detail by way of the following examples and drawing figures, to which, however, it shall not be restricted.

## 25       **Examples**

### **Example 1: Immunization of immunocompetent mice and generation of anti-FIX/IXa antibody secreting hybridoma cells**

Groups of 1-3 normal immunocompetent 5-8 week old  
30 Balb/c mice were immunized with 100 $\mu$ g antigen (100 $\mu$ l doses) via the intraperitoneal (i.p.) route. In a typical experiment, mice were inoculated with either

recombinant human coagulation factor (F) IX (Benefix<sup>TM</sup>),  
human activated FIX $\alpha$  (Enzyme Research Laboratories,  
Lot: FIX $\alpha$  1190L) or human FIX $\alpha\beta$  (Enzyme Research  
Laboratories, Lot: HFIXA $\alpha\beta$  1332 AL,) adjuvanted with  
5 Al(OH)<sub>3</sub> or KFA.

Individual mice were boosted at various times with  
100 $\mu$ g antigen (100 $\mu$ l doses, i.p) and sacrificed two days  
later. Spleen cells were removed and fused to P3 X63-Ag8  
6.5.3 myeloma cells essentially as described by Lane et  
10 al., 1985 (J. Immunol. Methods, Vol. 81, pp. 223-228).  
Each fusion experiment was individually numbered, i.e.  
#193, 195, 196 or 198.

Hybridoma cells were grown in 96 well plates on a  
macrophage feeder layer (app. 10<sup>5</sup> cells/ml) and selected  
15 in HAT-medium (RPMI-1640 medium supplemented with  
antibiotics, 10% FCS, Na-pyruvate, L-glutamine, 2-  
mercaptoethanol and HAT (HAT 100x: 1.0x10<sup>-2</sup>M  
hypoxanthine in H<sub>2</sub>O (136.1 mg/100ml H<sub>2</sub>O), 4.0x10<sup>-5</sup>M  
aminopterin in H<sub>2</sub>O (1.76 mg/100ml H<sub>2</sub>O) and 1.6x10<sup>-3</sup>M  
20 thymidine in H<sub>2</sub>O (38.7 mg/100ml H<sub>2</sub>O). Medium was first  
changed after 6 days and thereafter twice a week. After  
2-3 weeks HAT-medium was changed to HT-medium (RPMI-1640  
supplemented with antibiotics, 10%FCS, Na-pyruvate, L-  
glutamine, 2-mercaptoethanol and HT) and later on (after  
25 additional 1-2 weeks) to normal growth medium (RPMI-1640  
medium supplemented with 10%FCS, Na-pyruvate, L-  
glutamine and 2-mercaptoethanol) (see: HYBRIDOMA  
TECHNIQUES, EMBO, SKMB Course 1980, Basel).

In another set of experiments FIX deficient C57B16  
30 mice (Lin et al., 1997, Blood, 90:3962) were used for  
immunization and subsequent hybridoma production. Since  
FIX knockout (k.o.) mice do not express endogenous FIX,

the anti (a)-FIX antibody spectrum achievable is supposed to be different compared to normal Balb/c mice (due to lack of tolerance)

**Example 2: Assaying for FVIII-like activity in supernatants of anti-FIX/FIXa antibody secreting hybridoma cells**

In order to assay the FVIII-like activity of anti-FIXa antibodies secreted by hybridoma cells, the commercially available test-kit COATEST VIII:C/4® (Chromogenix) was employed. The assay was done essentially as described by the manufacturer with the following modifications:

To allow high throughput screening, the assay was downscaled to microtiter plate format. Briefly, 25µl aliquots of hybridoma supernatants were transferred to microtiter plate (Costar, #3598) wells and warmed to 37°C. Chromogenic substrate (S-2222), synthetic thrombin inhibitor (I-2581), factor (F) IXa and FX were reconstituted in sterile water and FIXa/FX was mixed with phospholipids according to the supplier's protocol. Per reaction, 50µl of the phospholipid/FIXa/FX solution were combined with 25µl CaCl<sub>2</sub> (25mM) and 50µl of the substrate/inhibitor cocktail. To start the reaction, 125µl of the premix were added to the hybridoma supernatant in the microtiter plates and incubated at 37°C. Absorbency at 405nm and 490nm of the samples was read at various times (30min to 12h) against a reagent blank (MLW, cell culture medium instead of hybridoma supernatant) in a Labsystems iEMS Reader MF™ microtiter plate reader. FVIII-like activity of the samples was calculated by comparing the absorbency of the samples against the absorbency of a diluted FVIII reference standard (IMMUNO AG # 5T4AR00) using GENESIS™ software.

The results of a screening for FVIII-like activity in hybridoma cell culture supernatants are shown in Fig.



1. Pre-selected clones derived from fusion experiments #193, #195 and #196 (see above) were examined in a chromogenic FVIII assay as described. Clones 193/M1, 193/N1 and 193/P1 are subclones derived from the master clone 193/C0 (see below). Master clone 195/10 was derived from fusion experiment #195 and clones 196/A0, 196/B0 and 196/C0 were derived from fusion experiment #196. In a typical screening experiment, approximately 1000 clones (in 96 wells) from a single fusion experiment were pre-screened for FVIII-like activity. Subsequently, selected clones were grown on a larger scale (3-5 ml supernatant) and re-analyzed in a chromogenic assay. As a negative control cell culture medium was assayed on each plate (MLW).

Wells either exhibiting high FVIII-like activity or substantial FVIII-like activity were subjected to subcloning procedures. The selection and subcloning process is exemplified for the screening and subcloning of an IgG producing cell line (i.e. 193/C0) but has been done exactly the same way for an IgM (i.e. 196/C0, see below, Fig. 5) producing clone.

The selection process was done by initially plating all hybridoma cell clones derived from a single fusion experiment on ten 96 well plates thereby creating the so called "master plates". Singular positions (wells) on a master plate usually contained more than one hybridoma cell clone (usually 3 to 15 different clones). Subsequently, the antibody secreted by only several thousand cells was tested. These cells grew under conditions suboptimal for antibody production, which is known to be best in dying cells. So the expected specific anti-FIX antibody concentration in the supernatant may be in the range of  $10^{-12}$  to  $10^{-14}$  M.

This explains why incubation periods had to be extended compared to standard FVIII assays.

Results of a screening for an IgG mediated FVIII-like activity in hybridoma cell culture supernatants of a master plate are shown in Fig. 2. Supernatants were examined in a chromogenic FVIII assay. Shown are the results derived from the fifth master plate of fusion experiment number #193 (Balb/c mice immunized with FIX $\alpha$ ). Absorbance was read after 4 hours of incubation at 37°C. Position ES was identified as exhibiting FVIII like activity significantly higher than the blank (MLW). This cell pool was designated 193/C0 and was further subcloned (Figure 3). As each well of the master plate contains more than one hybridoma cell clone, cells of a single positive well were expanded and plated at a calculated cell density of 2 - 0.2 cells/well on a 96 well plate. Again, the supernatants were tested for FVIII-like activity and positive positions were subjected to another round of subcloning. Typically three to four rounds of subcloning were performed with each clone displaying FVIII-like activity to obtain homogenous cell populations. Here the results of the chromogenic assay of the 193/C0 subclones are shown. Absorbance was read after a 4 hour incubation period at 37°C. Positions A6 and D5 exhibited substantial FVIII-like activity and were named 193/M1 and 193/P1, respectively. These two clones were subjected to another round of subcloning. As a negative control plain cell culture medium was assayed on each plate (MLW(H1)).

A comparison of chromogenic FVIII-like activity and FIX-ELISA reactivity of small scale (3 ml) hybridoma cultures is shown in Fig. 4. Before a decision was made whether a master clone (or subclone) was to be further

subcloned, clones were grown at a 3-5 ml scale and the supernatants were checked again. This graph shows the FIX specific ELISA results and the FVIII-like chromogenic activity of the master clone 193/C0 and all its subclones which were identified as positives and re-checked. Blanks (absorbency of the chromogenic reagent itself) were subtracted in the case of the ELISA as well as the chromogenic assay readings depicted here. Clone 193/M1 was subcloned and yielded clones 193/V2, 193/M2 and 193/U2. The other clones of the 2<sup>nd</sup> round came from 193/P1, 193/AB2 and 193/P2 were subcloned. 193/AF3, 193/AB3 and 193/AE3 are subclones of 193/AB2. The other clones of the 3<sup>rd</sup> round came from 193/P2. Finally 193/AF3 (→193/AF4), AE3 (→193/AE4, 193/AL4, 193/AN4 and 193/AO4) and 193/AD3 (→193/AG4, 193/AH4, 193/AD4, 193/AI4, 193/AK4) were subcloned.

From each fusion experiment, several (5-15) master clones (selected from the master plate) were identified and subjected to subcloning. After 3 rounds of subcloning, most of the cell lines were homogenous as demonstrated by ELISA and chromogenic activity analysis (see Fig. 4) as well as by cDNA sequence analysis. A specific master clone and all its subclones produce the same FIX/FIXa binding antibody. However, there are huge differences in the antibody protein sequences of clones derived from different master clones (see Example 11). Most hybridoma cell lines express antibodies from the IgG subclass (i.e. clones #193, #198, like 198/A1, 198/B1, 198/BB1). However, we were also able to select some clones expressing IgM antibodies.

The chromogenic activity of hybridoma supernatant of some important master clones and subclones was determined. Absorbance was measured after a 1h 30 min

and 3h 30 min incubation period at 37°C (Fig. 5). In contrast to all the clones from the 193<sup>rd</sup> fusion, clone 196/C0 and its subclone 196/AP2 produced a FIX/FIXa-specific IgM antibody that gave a strong chromogenic activity even after a short period of incubation.

The following cell lines have been deposited with the European Collection of Cell Cultures (ECACC) in accordance with the Budapest Treaty: 98/B1 (ECACC No. 99090925); 198/A1 (ECACC No. 99090924); 198/BB1 (ECACC No. 99090926); 193/AO (ECACC No. 99121614); 196/C4 (ECACC No. 99121615); 198/D1 (ECACC No. 99121616); 198/T2 (ECACC No. 99121617); 198/G2 (ECACC No. 99121618); 198/AC1 (ECACC No. 99121619); and 198/U2 (ECACC No. 99121620).

To do a more in depth analysis of the biochemical properties of certain antibodies, homogenous hybridoma cell lines expressing different antibodies with FVIII-like activity were expanded and used to express the antibody in question on a larger scale (100-1000 ml). These antibodies were affinity purified (see Example 3) prior to being used in further experiments.

**Example 3: Factor IX/FIXa( $\alpha$ , $\beta$ ) binding properties of antibodies exhibiting FIX/FIXa activating activity**

Factor IX and the two activated forms of FIX, FIX $\alpha$  and FIX $\alpha\beta$  (FIX/FIXa( $\alpha$ , $\beta$ )) were diluted in TBS (25mM Tris HCl, 150mM NaCl, pH 7.5) to a final concentration of 2 $\mu$ g/ml. Nunc Maxisorp ELISA plates were coated with 100 $\mu$ l FIX/FIXa( $\alpha$ , $\beta$ ) solution according to standard procedures (4°C, overnight) and washed several times with TBST (TBS, 0.1% (v/v) Tween 20). 50 $\mu$ l hybridoma supernatant was diluted 1:1 with 50 $\mu$ l TBST/2%BSA and added to the coated ELISA plate. After an incubation

period of 2h at room temperature (RT), plates were washed 4 times with TBST and incubated (2h, RT) with 100µl/well of a 1:25000 dilution (in TBST/1%BSA) of an anti-mouse IgG (Fc-specific) peroxidase conjugated antibody (Sigma, #A-0168). Wells were washed 5 times with TBST and finally stained with 100µl freshly prepared staining solution (10ml 50mM sodium citrate, pH 5 supplemented with 100µl OPD (60mg OPD/ml) and 10µl 30% H<sub>2</sub>O<sub>2</sub>). The reaction was stopped by the addition of 50ml H<sub>2</sub>SO<sub>4</sub> and the optical density recorded at 492nm and 620nm in a Labsystems iEMS Reader MF™ microtiter plate reader employing GENESIS™ software.

In certain cases, instead of an anti-mouse IgG ELISA, an anti-mouse IgM ELISA was carried out.

#### **Purification of mouse-IgG from hybridoma cell culture supernatants**

Hybridoma supernatant (100-500 ml) was supplemented with 200 mM Tris/HCl buffer (pH 7.0) and solid NaCl to give final concentrations of 20 mM Tris and 3M NaCl, respectively. The supernatant was then clarified by centrifugation at 5500 x g for 10 minutes. A 1 ml protein G affinity chromatography column (Protein G Sepharose Fast Flow, Amersham-Pharmacia) was washed with 15 ml 20 mM Tris/Cl pH 7.0 and afterwards equilibrated with 10 ml of 20 mM Tris/Cl buffer pH 7.0 containing 3M NaCl. The hybridoma supernatant containing 3M NaCl was then loaded onto the column by gravity. The column was washed with 15 ml of 20 mM Tris/Cl buffer, pH 7.0, containing 3M NaCl. Bound IgG was further eluted with 12 ml glycine/HCl buffer pH 2.8 and 1 ml fractions were collected. 100µl of 1M Tris pH 9.0 were added to each fraction for neutralization. Fractions containing the

IgG were identified by mixing 50 $\mu$ l with 150 $\mu$ l of a staining solution (BioRad concentrate, 1:5 diluted with water) in wells of a microplate. Positive fractions were pooled, concentrated to 1 ml in an ultrafiltration concentrator device (Centricon Plus 20, Amicon) according to the manufacturer. The concentrate was diluted with 19 ml TBS (20 mM Tris/Cl buffer pH 7.0 containing 150mM NaCl) and again concentrated to 1 ml. The diluting-concentrating step was repeated for two more times in order to bring IgG into TBS.

**Purification of mouse-IgM from hybridoma cell supernatants**

100-500 ml of hybridoma cell culture supernatant were concentrated to 5-10 ml either with an ultrafiltration concentrator device (Centricon Plus 20, Amicon) according to the manufacturer or by ammonium sulfate precipitation (40% saturation, 0°C) and redissolving the precipitate with 5-10 ml of TBS. In either case the concentrate was dialyzed against 20mM Tris Cl buffer pH 7.4 containing 1.25M NaCl and further concentrated to 1 ml in a Centricon Plus 20, (Amicon) ultrafiltration device. IgM was purified from this concentrate with the ImmunoPure IgM Purification Kit (Pierce) according to the manufacturer. Fractions collected during elution from the maltose binding protein-column were tested for IgM, pooled, concentrated and brought into TBS as described for IgG.

**Determination of IgG concentrations in purified preparations**

Total IgG content 280nm - extinction of appropriate dilutions were measured. E280 = 1.4 corresponds to 1 mg/ml protein.

**Factor IXa specific IgG (quantitative ELISA)**

Wells of a microplate (Nunc Maxisorp) were incubated with 2µg/ml factor IXa diluted in TBS (25mM Tris/HCl pH 7.5 containing 150mM NaCl) overnight at 4°C. Wells were washed four times with TBST (25mM Tris/HCl pH 7.5 containing 150mM NaCl and 0.1% (v/v) Tween 20). As a standard monoclonal AB the HIX1 anti-FIX (accurate) was used. Standard and samples were diluted in TBST containing 2%(w/v) BSA. The standard dilution series and appropriate dilutions of the samples were incubated on the ELISA-plate for 2 hours at room temperature. Plates were washed 4 times with TBST and incubated (2h, RT) with 100µl/well of a 1:25000 dilution (in TBST/1%BSA) of an anti-mouse IgG (Fc-specific) peroxidase conjugated antibody (Sigma, #A-0168) FIXa. Wells were washed 5 times with TBST and finally stained with 100µl freshly prepared staining solution (10ml 50mM sodium citrate, pH 5 supplemented with 100µl OPD (60mg OPD/ml) and 10µl 30% H<sub>2</sub>O<sub>2</sub>). The reaction was stopped by the addition of 50ml H<sub>2</sub>SO<sub>4</sub> and after 30 minutes the optical density was recorded at 492nm and 620nm in a Labsystems iEMS Reader MFTM microtiter plate reader employing GENESIS<sup>TM</sup> software.

**Example 4: Anti-FIX/FIXa antibodies exhibiting FVIII-like activity in a chromogenic FVIII assay**

Several anti-FIX/FIXa antibody producing hybridoma clones were subcloned up to four times and the resulting monoclonal hybridoma cell line used to produce monoclonal antibody containing supernatant. IgG isotype antibodies derived from these supernatants were purified over affinity columns and dialyzed against TBS (see above). IgM antibodies were used as unpurified supernatant fractions. The following experiments were

done with two sets of representative antibodies: 193/AD3 and 198/AC1/1 (IgG isotype, the antibody 198/AC1/1 is a preparation from the parent 198/AC1 hybridoma clone, i.e. that a (frozen) vial containing 198/AC1 cells is  
5 cultivated and antibodies are produced. The supernatant is then used for these experiments.) and 196/AF2 and 196/AF1 (IgM isotype) (Fig. 6A and Fig. 6B). Briefly, 25µl aliquots of monoclonal antibody containing sample (unpurified hybridoma supernatant or, where indicated, a  
10 certain amount of FIX specific antibody) were transferred to microtiter plate wells and warmed to 37°C. Chromogenic substrate (S-2222), synthetic thrombin inhibitor (I-2581), factor (F) IXa and FX were reconstituted in sterile water and FIXa/FX was mixed  
15 with phospholipids according to the supplier's protocol. Per reaction, 50µl of the phospholipid/FIXa/FX solution were combined with 25µl CaCl<sub>2</sub> (25mM) and 50µl of the substrate/inhibitor cocktail. To start the reaction, 125µl of the premix were added to the monoclonal  
20 antibody solution in the microtiter plates and incubated at 37°C. Absorbance at 405nm and 490nm of the samples was read at various times (5min to 6h) against a reagent blank (cell culture medium instead of hybridoma supernatant) in a Labsystems iEMS Reader MF™ microtiter  
25 plate reader using GENESIS™ software.

The time course of FVIII-like activity exhibited by monoclonal antibodies 193/AD3 (IgG isotype) and 196/AF2 (IgM isotype) compared to human FVIII (12 and 16mU/ml), TBS and to cell culture medium is shown in Fig. 6A.  
30 After a lag phase, both antibodies give rise to chromogenic substrate cleavage, as judged by the



increasing optical density measurable at 405nm wavelength.

The time course of FVIII-like activity exhibited by monoclonal antibodies 198/AC1/1 (IgG isotype, 10µg/ml) and 196/AF1 (IgM isotype, unpurified supernatant) compared to human FVIII (16mU/ml) and 10µg/ml of mouse IgG is shown in Fig. 6B. After a lag phase, both antibodies give rise to chromogenic substrate cleavage, as judged by the increasing optical density measurable at 405nm wavelength.

**Example 5: FVIII-like activity exhibited by anti-FIX/FIXa-antibodies generates factor Xa and is phospholipid, FIXa/FX and  $\text{Ca}^{2+}$  dependent.**

Factor VIII activity is usually determined with a chromogenic assay and/or an APTT-based clotting assay. Both types of assays rely on FVIIIa/FIXa-mediated factor Xa generation. In the case of a chromogenic FVIII assay, the factor Xa produced will subsequently react with a chromogenic substrate, which can be monitored spectroscopically, e.g., in an ELISA reader. In an APTT based clotting assay free factor Xa will assemble with FVa on a phospholipid surface in the so-called prothrombinase complex and activate prothrombin to thrombin. Thrombin in turn gives rise to fibrin generation and finally to clot formation. Central to the two assay systems is generation of factor Xa by the FVIIIa/FIXa complex.

To demonstrate that the FVIII-like activity exhibited by anti-FIX/FIXa-antibodies indeed generates factor Xa, the following experiment was carried out. Several 25µl aliquots of unpurified hybridoma supernatant 196/AF2 (IgM isotype) were transferred to microtiter plate wells and warmed to 37°C. As a positive control, 16mU of

Recombinate<sup>TM</sup> were diluted into hybridoma medium (196 HM 007/99) and treated exactly the same way as the hybridoma supernatant. As a negative control, plain hybridoma medium was used. Chromogenic substrate (S-2222), synthetic thrombin inhibitor (I-2581), factor IXa and FX were reconstituted in sterile water and FIXa/FX was mixed with phospholipids according to the supplier's protocol. Pefabloc Xa®, a factor Xa specific proteinase inhibitor (Pentapharm, LTD), was reconstituted with water to a final concentration of 1mM/l. Per reaction, 50µl of the phospholipid/FIXa/FX solution were combined with 25µl CaCl<sub>2</sub> (25mM) and 50µl of the substrate/thrombin-inhibitor cocktail. To start the reaction, 125µl of the premix were added to the samples in the microtiter plates and incubated at 37°C. Where indicated, 35µM Pefabloc Xa® were added. Absorbance at 405nm and 490nm was read at various times (every 5 minutes to 6h) against a reagent blank (cell culture medium) in a Labsystems iEMS Reader MF<sup>TM</sup> microtiter plate reader employing the GENESIS<sup>TM</sup> software.

The results of the factor IXa stimulation by the FVIII-like activity exhibited by the IgM anti- FIX/FIXa-antibody 196/AF2 in generating factor Xa as judged by the readily measurable cleavage of the chromogenic substrate S-2222 (compare "16mU FVIII" and "196/AF2") is shown in Fig. 7A. Factor Xa activity is effectively blocked by the FXa specific inhibitor "Pefabloc Xa®" (compare "196/AF2" versus "196/AF2 35µM Pefabloc Xa®") indicating that indeed FXa was generated.

The same experiment was performed using purified IgG preparations of clone 198/AM1 (Fig. 7B). Purified IgG was diluted in TBS to a final concentration of

0,4mg/ml and 25 $\mu$ l (i.e. a total of 10 $\mu$ g), transferred to microtiter plate wells and warmed to 37°C. As a positive control, 6mU plasma- derived FVIII was used. 10 $\mu$ g unspecific mouse IgG (Sigma, I-5381) served as a  
5 negative control. The assay was performed as described above.

Further experiments show the factor IXa stimulation by the FVIII-like activity exhibited by the IgG anti-FIX/FIXa-antibody 198/AM1 generates factor Xa as judged  
10 by the readily measurable cleavage of the chromogenic substrate S-2222 (Fig. 7B). Again factor VIII and antibody 198/AM1 generate FXa which is effectively blocked by the FXa specific inhibitor "Pefabloc Xa®". As a negative control, unspecific mouse IgG (Sigma, I5381)  
15 was assayed.

In another set of experiments, the dependence of the FVIII-like activity of either purified anti-FIX/FIXa-antibodies (IgM, Fig.8A) or of unpurified antibodies derived from cell culture supernatants (IgG, Fig. 8B) on the presence of phospholipids (PL), FIXa/FX and Ca<sup>2+</sup> was demonstrated. Mouse IgG was used as a control (Fig. 8C). Factor VIII-like activity was assayed essentially as described above. When indicated, either the FIXa/FX mixture, the PL or Ca<sup>2+</sup> was omitted from the  
25 reaction. Absorbency at 405nm and 490nm of the samples was read at various times against a reagent blank (buffer instead of purified antibody) in a Labsystems iEMS Reader MF™ microtiter plate reader. The results are shown in Fig. 8A, Fig. 8B and Fig. 8C.

30 The dependence of the FVIII-like activity of purified anti-FIXa-antibody 198/AC1/1 (IgG isotype, concentration used throughout the assay was 10 $\mu$ g/ml) on

the presence of phospholipids (PL), FIXa/FX and  $\text{Ca}^{2+}$  is further shown in Fig. 8A. As is easily recognizable, only the complete assay, including antibody, PL,  $\text{Ca}^{2+}$ , and FIXa/FX gives rise to a reasonable FXa generation.

- 5 The dependence of the FVIII-like activity of cell culture supernatant containing unpurified IgM isotype anti-FIX/FIXa-antibody (196/AF1) on the presence of phospholipids, FIXa/FX and  $\text{Ca}^{2+}$  is shown in Fig. 8B.

- Again, as already shown for the purified IgG preparation (Fig. 8A), antibody 198/AC1/1, only the complete assay, including PL,  $\text{Ca}^{2+}$ , FIXa/FX, will give a reasonable amount of FXa generation. To demonstrate the specificity of the reaction, total IgG prepared from normal mouse plasma was assayed under the same conditions as above. The results are shown in Fig. 8C. No FVIII-like activity could be detected. There is, as expected, no activity detectable in the absence of phospholipids, FIXa/FX and  $\text{Ca}^{2+}$ . All experiments were done in a microtiter plate and the OD405 was scanned every 5 minutes for 6h.

**Example 6: Certain anti-FIX/FIXa-antibodies are procoagulant in the presence of FIXa**

- During normal hemostasis, FIX becomes initially activated either by the tissue factor (TF)/factor VIIa pathway or later on by activated factor XI (FXIa). Subsequent to its activation, FIXa associates on the platelet surface in a membrane bound complex with activated FVIII. Factor IXa by itself has little or no enzymatic activity towards FX, but becomes highly active in the presence of FVIIIa. To demonstrate that certain anti-FIX/FIXa antibodies have FVIII-like activity and hence are procoagulant in a FVIII deficient human plasma, the following experiment was carried out.

Different amounts of antibody 193/AD3 or mouse IgG (as a control) were used in a standard aPTT based one stage clotting assay. Briefly, 100 $\mu$ l of antibody-containing samples were incubated with 100 $\mu$ l of FVIII deficient plasma (DP) and with 100 $\mu$ l of DAPTTIN (PTT Reagent for determining activated Thromboplastin Time; IMMUNO AG) reagent, in a KC10A clotting analyzer. Where indicated, a total amount of 50ng activated FIX was included in the reaction mixture. After a 4 minute incubation, the reaction was started by the addition of 100 $\mu$ l CaCl<sub>2</sub> (25mM). The results are shown in Table 1 and Fig. 9.

clotting time (sec)		
$\mu$ g AB	193/AD3	mouse IgG
	50ng FIXa	50ngFIXa
9	101.6	102.5
4.5	95.6	103.2
2.25	93.1	103.2
1.8	93.7	101.9
1.35	91.4	103.4
0.9	94.4	102.2
0.45	98.1	101.9
0.34	97.1	103.9
0.23	99.3	103.7

Table 1: Clotting times of FVIII deficient plasma in an APTT based clotting assay employing various amounts of procoagulant (193/AD3) and control antibody (mouse IgG) in the presence of 50ng activated FIX (0.01UFIX). The molar ratio of antibody in the reaction and activated FIX is 10:1. The molar ratio between antibody and total FIX (FIX and FIXa, assuming that human FVIII deficient

plasma contains 1U (5 $\mu$ g) FIX) varies between 6:1 (9 $\mu$ g antibody in reaction) and 1:6 (0.23 $\mu$ g antibody in reaction). At the optimal shortening of the clotting time, the molar ratio between antibody and total FIX is 1:1. The clotting time without the addition of FIXa is in the range of 120 seconds.

Fig. 9 is a graphical representation of the clotting times of FVIII deficient plasma in an aPTT based clotting assay employing various amounts of procoagulant (193/AD3) and control (mouse IgG) antibody in the presence of 50ng activated FIX. There is a clear dose-dependent reduction of the clotting time in samples supplemented with antibody 193/AD3. These results imply that antibody 193/AD3 is procoagulant in the presence of FIXa.

**Example 7: Anti-FIX/FIXa-antibodies are procoagulant in the presence of FVIII inhibitors and FIXa**

A severe complication of the standard FVIII substitution therapy is the development of alloantibodies directed against FVIII, leading to FVIII neutralization and a condition where the patient's blood will not clot.

To demonstrate that certain anti-FIXa-antibodies have FVIII-like activity even in the presence of FVIII inhibitors, the following experiment was carried out. Different amounts of antibody 193/AD3 or, as a control, mouse IgG were used in a standard APTT based one-stage clotting assay. Briefly, 100 $\mu$ l antibody samples were incubated with either 100 $\mu$ l of FVIII deficient plasma (Fig.10A) or FVIII inhibitor plasma (inhibitor potency 400BU/ml), Fig.10B) as well as with 100 $\mu$ l of DAPTTIN

reagent, in a KC10A clotting analyzer. In addition, a total amount of 50ng activated FIXa was included in the reaction mixture. After a 4 minute incubation, the reaction was started by the addition of 100μl CaCl<sub>2</sub> (25mM). To ensure equal conditions, the experiments employing FVIII deficient plasma and FVIII inhibitor plasma were done side by side. The results are shown in Fig. 10A and 10B. As already shown in Example 6, there is a clear dose-dependent reduction of the clotting time in samples supplemented with antibody 193/AD3 in the presence of FVIII inhibitors.

**Example 8: Anti-FIX/FIXa-antibodies are procoagulant in the presence of defective FVIII and FIXa**

To demonstrate that certain anti-FIXa-antibodies have FVIII-like activity in the presence of defective FVIII, the following experiment may be carried out. Increasing amounts of antibody 193/AD3 or, as a control, mouse IgG are used in a standard aPTT-based one stage clotting assay. In this clotting assay, a hemophilia A patient's plasma having very low clotting activity due to the presence of defective FVIII (DF8) is used. Briefly, 100μl antibody samples are incubated with either 100μl of DF8 plasma or FVIII deficient plasma as well as with 100μl of DAPTTIN reagent, in a KC10A clotting analyzer. In addition, a total amount of 50ng activated FIXa is included in the reaction mixture. After a short incubation, the reaction will be started by the addition of 100μl CaCl<sub>2</sub> (25mM). To ensure equal conditions, the experiment employing FVIII deficient plasma and DF8 plasma is done side by side.

**Example 9: Anti-FIX/FIXa-antibodies with procoagulant activity in the presence of FIXa distinguish between human and bovine FIXa**

FIX/FIXa specific monoclonal antibodies selected  
5 from the 198<sup>th</sup> fusion experiment were purified from the  
respective hybridoma supernatant and quantified as  
described in Example 3. These antibodies were analyzed  
in a modified one-stage clotting assay (as described in  
Example 6) and some showed procoagulant activity.

10 The chromogenic activity of these antibody  
preparations was measured in the following FXa  
generation kinetic assay: 10µg of monoclonal antibody  
(in 25µl) were transferred to microtiter plate wells and  
warmed to 37°C. Chromogenic substrate (S-2222),  
15 synthetic thrombin inhibitor (I-2581), factor IXa and FX  
were reconstituted in sterile water and FIXa/FX (both  
bovine) were mixed with phospholipids according to the  
supplier's protocol. Per reaction, 50µl of the  
phospholipid/FIXa/FX solution were combined with 25µl  
20 CaCl<sub>2</sub> (25mM) and 50µl of the substrate/inhibitor  
cocktail. To start the reaction, 125µl of the premix  
were added to the monoclonal antibody solution in the  
microtiter plates and incubated at 37°C. Absorbance at  
405nm and 490nm of the samples was read at various times  
25 (5min to 2h) against a reagent blank (25ml TBS instead  
of monoclonal antibodies) in a Labsystems iEMS Reader  
MF<sup>TM</sup> microtiter plate reader using GENESIS<sup>TM</sup> software.  
In parallel, the same reactions were performed except  
that 50ng human FIXa were added per reaction. Those  
30 antibodies that showed procoagulant activity had no  
chromogenic activity in the case of bovine FIX, but  
displayed high activity when human FIXa was present.



Fig. 11 shows the time course of the FVIII-like activity exhibited by the monoclonal antibodies 198/A1, 198/B1 and 198/AP1 with (+) and without (-) addition of 50ng human FIXa $\beta$ . Non-specific polyclonal mouse IgG was used as a control. 198/A1 and 198/B1 show procoagulant activity (similar as 193/AD3 in example 6) whereas 198/AP1 does not. Antibody 198/BB1 had the same activity pattern (data not shown).

Further monoclonal antibodies selected from the 198<sup>th</sup> fusion experiment include 198/D1 (ECACC NO. 99121616), 198/T2 (ECACC No. 99121617), 198/G2 (ECACC No. 99121118), 198/U2 (ECACC No. 99121620).

**Example 10 : Structure and procoagulant activity of antibody derivatives derived from anti-FIX/FIXa-antibodies; Subcloning antibody variable domains from hybridoma cell lines 193/AD3, 193/K2, 198/A1 and 198/B1 (clone AB2)**

Cloning procedure: Messenger RNA was prepared from 1x10<sup>6</sup> hybridoma cells of the respective cell line (either 193/AD3, 193/K2, 198/A1 or 198/B1 (clone AB2)) employing the "QickPrep® Micro mRNA Purification Kit" (Pharmacia) according to the manufacturer's instructions. The corresponding cDNA was produced by retro transcription of mRNA using the "Ready-To-Go-You-Prime-First-Strand Beads kit" (Pharmacia) according to the manufacturer's instructions. Heavy and light chain encoding sequences were converted to the corresponding cDNA employing a set of primers. To reverse transcribe heavy chain-specific mRNA (VH), an equimolar mixture of the oligonucleotides MOCG1-2FOR (5' CTC AAT TTT CTT GTC CAC CTT GGT GC 3') (SEQ.ID.NO. 1), MOCG3FOR (5' CTC GAT TCT CTT GAT CAA CTC AGT CT 3') (SEQ.ID.NO. 2) and MOCMFOR (5' TGG AAT GGG CAC ATG CAG ATC TCT 3')

(SEQ.ID.NO. 3) was used (RTmix1). In the same reaction tube, light chain-specific cDNA (VL) was synthesized using primer MOCKFOR -(5' CTC ATT CCT GTT GAA GCT CTT GAC 3') (SEQ.ID.NO. 4).

5       The coding sequences for VH were amplified by PCR using the primer-sets depicted in Fig. 12 and the specific cDNA, derived from the reverse transcription mixture (RTmix1) described above, as the template. VK-chain genes were amplified using the primer sets  
10       depicted in Fig. 13 and also employing Rtmix1 as a template. The VH-PCR product was cleaved SfiI-AscI and inserted into SfiI-AscI digested vector pDAP2 (GeneBank accession no.: U35316). The pDAP2-VH constructs obtained thereby were named pDAP2-193AD3/VH, pDAP2-198A1/VH,  
15       pDAP2-198AB2/VH (derived from antibody 198/B1) and pDAP2-193/K2/VH, respectively. The plasmids were subsequently cleaved with AscI-NotI and the corresponding AscI-NotI digested VK-gene PCR product was inserted. The resultant vectors were designated pDAP2-  
20       193/AD3scFv, pDAP2-198/A1scFv, pDAP2-198/AB2scFv (derived from antibody 198/B1) and pDAP2-193/K2scFv and code for the VH-gene and the VL-gene of the monoclonal antibodies 193/AD3, 198/A1, 198/AB2 (derived from  
25       antibody 198/B1) and 193/K2. Heavy and light chains are linked by the coding sequence for an artificial, flexible linker (G<sub>4</sub>SGGRASG<sub>4</sub>S; Engelhardt et al., 1994) and enables expression of the scFv variant of the respective antibody.

30       In Fig. 14, the DNA and the deduced protein sequence of the scFv derived from the hybridoma cell line 193/AD3 are depicted. Nucleotides 1 to 357 code for the heavy chain variable domain, nucleotides 358 to 402 code for the artificial flexible linker and nucleotides

403 to 726 code for the light chain variable region. The protein sequence of the CDR3 region of the heavy chain has the sequence YGNSPKGFAY (SEQ.ID.NO. 5) and is given in bold letters. The artificial linker sequence  
5 (G<sub>4</sub>SGGRASG<sub>4</sub>S) is shown.

In Fig. 15, the DNA and the deduced protein sequence of the scFv derived from the hybridoma cell line 193/K2 is shown. Nucleotides 1 to 363 code for the heavy chain variable domain, nucleotides 364 to 408 code  
10 for the artificial flexible linker, and nucleotides 409 to 747 code for the light chain variable region. The protein sequence of the CDR3 of the heavy chain has the sequence DGGHGYGSSFDY (SEQ.ID.NO. 6), and is given in bold letters. The artificial linker sequence  
15 (G<sub>4</sub>SGGRASG<sub>4</sub>S) is show.

In Fig. 16, the DNA and the deduced protein sequence of the scFv derived from the hybridoma cell line 198/AB2 (derived from antibody 198/B1) are depicted. Nucleotides 1 to 366 code for the heavy chain  
20 variable domain, nucleotides 367 to 411 code for the artificial flexible linker, and nucleotides 412-747 code for the light chain variable region. The protein sequence of the CDR3 region of the heavy chain has the sequence EGGGFTVNWYFDV (SEQ.ID.NO. 7) and is given in  
25 bold letters. The artificial linker sequence (G<sub>4</sub>SGGRASG<sub>4</sub>S) is also shown.

In Fig. 17, the DNA and the deduced protein sequence of the scFv derived from the hybridoma cell line 198/A1 are depicted. Nucleotides 1 to 366 code for the heavy chain  
30 variable domain, nucleotides 367 to 411 code for an artificial flexible linker, and nucleotides 412-747 code for the light chain variable region. The protein sequence of the CDR3 region of the heavy chain has the

sequence EGGGYVNWYFDV (SEQ.ID.NO.8 ) and is given in bold letters. The artificial linker sequence (G<sub>4</sub>SGGRASG<sub>4</sub>S) is also shown.

**Example 11: Procoagulant activity of peptides  
5 derived from CDR3 regions of anti-FIX/FIXa-antibodies**

In principle, the antibody molecule can be envisioned as a biological device for the presentation of a combinatorial array of peptide elements in three dimensional space (see Gao et al., 1999, PNAS, 96:6025).  
10 Therefore, an antibody (or an antibody derivative, e.g. scFv, Fab, etc.) can be used either as a tool for the detection of functionally important domains of a specific target protein, or on the other hand, for the delineation of amino acid sequences, specifically  
15 mediating certain interactions, i.e. activating or enhancing the activity of FIXa towards the physiological substrate FX. The latter process has led to the evaluation of a number of heavy chain CDR3 region (CDR3<sub>H</sub>) derived peptide sequences as FIXa enhancing  
20 agents.

Enhancing the procoagulant activity of peptides which exhibit such activity may be accomplished through sequence variation within the peptide regions critical for mediating the FIXa activity enhancement. As a  
25 possible step towards peptide sequences with enhanced procoagulant activity, the binding site of an antibody, i.e. 198/A1 or 198/B1, on the FIXa molecule is mapped by employing sequence comparison analyses, competitive binding assays, Western blot analyses and competitive  
30 ELISA analyses. Since the crystal structure of FIX is known, molecular modeling is subsequently used to

improve the fitting of i.e. 198/B1 derived peptides in the 198/B1 binding site on human FIXa.

On the other hand, methodical mutational analysis of a given peptide sequence such as 198/A1 or 198/B1 CDR3<sub>H</sub> derived peptide sequences by, e.g., "alanine scanning mutational analysis" allows for the identification of peptide residues critical for procoagulant activity. Another way to improve the activity of a certain peptide sequence is the use of peptide libraries combined with high throughput screening.

The antigen binding site of an antibody is derived from the juxtaposition of the six "complement determining regions (CDR's)" at the N-terminal end of the VL-HL dimer (or Fv region). The contribution of a single CDR to the antibody specificity for a given antigen may vary considerably, but in general it is thought that the CDR3 region of the heavy chain (CDR3<sub>H</sub>) is of special influence, i.e. the particular protein sequence of CDR3<sub>H</sub> region may be highly important for antigen recognition. The length of CDR3<sub>H</sub> regions has been reported to vary considerably and is in the range of 4-25 amino acids (Borrebaeck, p.16).

An example of a methodical mutational analysis of peptide sequences is given below. To improve the solubility/procoagulant efficacy of peptides derived from the CD3-region of anti FIX/FIXa antibodies, the N-terminal as well as the C-terminal amino acid sequences were changed. In addition, a series of mutated peptides was constructed and analyzed.

The principle of such a study is exemplified by a series of peptides derived from CDR3<sub>H</sub> region of antibodies 198/A1 and 198/B1. The original peptide A1 (see table

2) is derived from the CDR3<sub>H</sub> region of antibody 198/A1 and peptide B1 is derived from the CDR3<sub>H</sub> region of antibody 198/B1, respectively (see example 10, Fig. 16 and 17). The term "scrambled version" means that a peptide has the same amino acids but in random order.

Peptide	Sequence	Amino-acids	MW (D)	pI	Remark
A1	EGGGYYVNWYFDV (SEQ.ID.No. 9)	(13aa)	1569	7,2	Decreased solubility
A1/1	VYGFGWGYEVNDY (SEQ.ID.No. 10)	(13aa)	1569	7,1	Scrambled version of A1,
A1/2	EEEEGGGYVNWYFDEEE (SEQ.ID.No. 11)	(18aa)	2244	5,8	Acidic pI, soluble,
A1/3	RRREGGGYYVNWYFDRRR (SEQ.ID.No. 12)	(18aa)	2407	9,9	Basic pI, soluble,
A1/4	EYGEYGEVNEYDEFWE (SEQ.ID.No. 13)	(18aa)	2244	5,8	Scrambled version of A1/2
A1/5	VRYSRNRWGYRGFRGDE (SEQ.ID.No. 14)	(18aa)	2407	9,9	Scrambled version of A1/3
A1/3-scr3	RRRGEYGVYWNGDFYRRR (SEQ.ID.No. 15)	(18aa)	2407	9,9	Scrambled version of A1/3
A1/3-Rd	RdRdRdEGGGYYVNWYFDRdRdRd (SEQ.ID.No. 16)	(18aa)	2407	9,9	Peptide A1/3 but substitute D-Arg for L-Arg
A1/3-Rd-srmb	RdRdRdGEYGVYWNGDFYRdRdRd (SEQ.ID.No. 17)	(18aa)	2407	9,9	Scrambled version of A1/3-Rd

Table 2

- 10 List of a series of antibody 198/A1 derived peptides. Listed are the length of the peptide (aa, amino acids #), the calculated molecular weight (MW, in Dalton (D)) and the statistical isoelectric point (pI). D-Arg is

abbreviated as Rd.

In a first series of experiments we improved the solubility of the original CDR3<sub>H</sub> peptide sequence (A1; EGGGYVNWYFDV) by removing the C-terminal Val residue and adding several charged residues at the N- as well as the C-terminal end of the peptide. The resulting peptides, A1/2 (acidic pI), A1/3 (basic pI) and their respective scrambled versions A1/4, A1/5 and A1/3scr3 were readily soluble in a variety of buffer systems at physiological pH.

To analyze the FVIII-like (FIXa activating) activity of the peptides, an assay system based on a commercial available FVIII assay was developed (see examples 2 and 4). The basic principle is, that without a cofactor, FIXa will have very limited activity towards its natural substrate FX. Only in the presence of a substance having FIXa activation properties, i.e. FVIII or a substance exhibiting FVIII-like activity, a substantial amount of FXa is produced by cleavage of FX through the FIXa/activator complex. The amount of FXa generated is monitored by cleavage of a chromogenic substrate. The principle of the revised chromogenic assay is described for two representative peptides: A1/3 and A1/5 (Table 2). Briefly, 25µl aliquots of peptide stock solution (in imidazole buffer (IZ) 50mM imidazole, 100mM NaCl, pH7.2) were transferred to microtiter plate wells and warmed to 37°C. Chromogenic FXa substrate (S-2222), synthetic thrombin inhibitor (I-2581), bovine FIXa and bovine FX were reconstituted in sterile water and FIXa/FX mixed with phospholipids according to the supplier's protocol.

Since the peptides do not react with bovine FIXa, (which comes as a mixture with bovine FX in the Test Kit) 2.9nM (in most cases 2.3nM) human FIXa (ERL) were added (see Example 11, Fig 19). Per reaction, 50µl of the phospholipid /FIXa/FX solution were combined with 25µl CaCl<sub>2</sub> (25mM) and 50µl of the substrate/inhibitor cocktail. To start the reaction, 125µl of the premix were added to the peptide solution in the microtiter plate and incubated at 37°C. Absorbance at 405nm and 490nm of the samples was read at various times (5 min to 2h) against a reagent blank in a Labsystems iEMS Reader MF™ microtiter plate reader using GENESIS™ software. The result of this experiment are shown in Example 11, Fig 18. Peptide A1/3 induced a readily measurable FXa generation in the presence of 2.9nM human FIXa, whereas the scrambled version A1/5 was inactive. In addition, the acidic peptide A1/2 as well as the scrambled versions A1/4 and A1/3-scr3 did not give any significant chromogenic activity when tested under comparable conditions (data not shown). To prove that the peptide A1/3 like the parental antibody 198/A1 does not react with bovine FIXa and FX the experiment shown in Fig. 19 was done. The peptide A1/3 was incubated as described above with (A1/3 (24µM), +hFIXa) and without (A1/3 (24µM), w/o hFIXa) 2.3nM human FIXa (hFIXa). In a control experiment we added plain dilution buffer (IZ) supplemented with 2.3nM hFIXa to the reaction mixture. As shown in Fig. 19, the reaction takes place only in the presence of human FIXa.

Fig. 18 demonstrates the chromogenic FVIII-like activity of peptide A1/3 in the presence of 2.9nM human FIXa (hFIXa). The scrambled version of peptide A1/3, peptide



A1/5 does not give rise to any FXa generation.

Fig. 19 demonstrates the dependence of the chromogenic FVIII-like activity of peptide A1/3 on the presence of human FIXa (hFIXa). In the absence of human FIXa,

- 5 peptide A1/3 does not give rise to any FXa generation. The buffer control, plain imidazole buffer is designated IZ.

The peptides were also analyzed for their potential to reduce the clotting time in a FVIII deficient plasma.

- 10 The aPTT based one stage clotting assay was essentially done as described (see example 6). Clotting times (time from starting the reaction to the "clot"-formation were compared either against FVIII, a buffer control (IZ) or a control peptide (scrambled version). The results of
- 15 two typical clotting experiments done with two different aPTT reagents (DAPTTIN and Pathromtin SL) are shown in table 3A and table 3B.

Exp. 1	peptide conc.	w/o FIXa sec	w/o FIXa sec	average sec	2.2nM FIXa sec	2.2nM FIXa sec	average sec
IZ	0	107,7	106,8	107	93,1	94,5	94
A1/3	15µM	78,2	77,1	78	59,3	59,9	60
	12,5µM	80,2	80,6	80	60,2	58,9	60
	7,5µM	97,8	97,9	98	73,1	72,7	73
	2,5µM	105,2	104,8	105	91,1	91	91
A1/3- scr3	15µM	122,5	122	122	106,1	105,5	106
	12,5µM	116	117,6	117	103,1	104,5	104
	7,5µM	114,2	113,9	114	100,8	100,6	101
	2,5µM	107,8	107,4	108	96,3	95,2	96
Exp. 2	peptide conc.	w/o FIXa sec	w/o FIXa sec	average (sec)	2.2nM FIXa sec	2.2nM FIXa sec	average (sec)
IZ	0	111	109,7	110	94,7	95,5	95
A1/3	12.5µM	83,6	85,5	85	56,7	56,7	57
	10µM	79,1	78,5	79	63,1	62,5	63
	7.5µM	100,1	100,5	100	71,6	73,9	73
	5µM	103,4	104,8	104	77	76	77
	2.5µM	110,1	108,9	110	88	88,8	88
	1,25µM	108,7	109,3	109	90,7	90,8	91

Table 3A. Clotting activity of peptides A1/3 and A1/3-scr (scrambled version of A1/3) in FVIII deficient plasma either in the presence or in the absence (w/o) of 2.2nM human FIXa. Shown are two independent representative experiments (Exp. 1 and Exp. 2). All clotting experiments have been done in duplicate. Given are the clotting times for the individual experiments and the average clotting time in seconds (sec). Experiments shown in table 3A have been done employing the aPTT reagent DAPTTIN (Baxter Hyland Immuno). Compared to the buffer control (IZ, imidazole buffer) the peptide A1/3 gave rise to a dose dependent reduction in the clotting time. The reduction in the clotting time

became much more pronounced by the addition of 2.2nM activated human FIX to the reaction mix. The scrambled version of peptide A1/3, A1/3-scr3 did not show any reduction of the clotting time. In fact, at concentrations above 2.5µM, the scrambled peptide became inhibitory and therefore prolonged the clotting time. Peptides A1/1, A1/2, A1/4 and A1/5 did not give any reduction in the clotting time indicating that they lack procoagulant activity (data not shown).

10

	Final conc.	w/o FIXa sec	w/o FIXa sec	average sec	2.2nM FIXa sec	2.2nM FIXa sec	average sec
IZ	0	131,8	132,1	132	107,9	108,7	108
FVIII	12,5mU/ml	68,9	69	69	52,9	53,6	53
	6,25mU/ml	77,8	77,9	78	58,6	58,9	59
A1/3							
	15µM	152,8	149,3	151	75,4	75,2	75
	10µM	135,7	134,6	135	76,2	79,8	78
	5µM	152,6	155,6	154	86,6	90,2	88
	1µM	138,3	138,8	139	103,7	105,9	105

Table 3B. Clotting activity of peptide A1/3 in FVIII deficient plasma when Pathromtin SL (DADE Behring) is used as an aPTT reagent. The experiments were done in duplicate, either in the presence or in the absence (w/o) of 2.2nM human FIXa. Given are the clotting times for the individual experiments and the average clotting time in seconds (sec). Factor VIII and imidazole buffer (IZ) were included as positive and negative control

20

respectively.

In contrast to the experiments shown in table 3A the experiments shown in table 3B have been done employing the aPTT reagent Pathromtin SL. In the presence of FIXa, the peptide A1/3 gave rise to a dose dependent reduction in the clotting time whereas in the absence of FIXa no reduction of the clotting time was detectable.

In another series of experiments we set out to improve the plasma stability (protection from, e.g., proteolytic degradation) of peptide A1/3. One approach was to substitute the N- and C-terminal L-Arg residues with D-Arg residues (exemplified by peptides A1/3-rd and A1/3-Rd-srmb). Peptides A1/3-rd and A1/3-Rd-srmb (scrambled version of the peptide) were then analyzed in a chromogenic as well as in the aPTT based clotting assay. These experiments revealed that exchanging the terminal L-Arg residues for D-Arg residues did not change the FVIII-like activity as measured in the chromogenic assay, indicating that chirality of the Arg-residues does not play a major role in chromogenic activity (Fig. 20). In addition, the aPTT based one-stage clotting activity, although somewhat reduced, was still easily detectable (Table 4).

	Peptide conc.	w/o FIXa sec	w/o FIXa, sec	average sec	2.2nM FIXa sec	2.2nM FIXa sec	average sec
I2	0	110	109,1	110	96	96	96
A1/3	15 $\mu$ M	77,8	78	78	56,1	55,5	56
	12,5 $\mu$ M	99,4	100,5	100	65	68	67
	10 $\mu$ M	104,4	104,5	104	72	73,2	73
	7,5 $\mu$ M	105,2	105,2	105	80,7	80,5	81
	5 $\mu$ M	108,4	107,7	108	89,7	88,3	89
	2,5 $\mu$ M	107,9	107,6	108	93,6	93,3	93
	1,25 $\mu$ M	106,7	107	107	94,4	95	95
A1/3-Rd	15 $\mu$ M	96,4	95,4	96	76,1	74,4	75
	12,5 $\mu$ M	98	98,6	98	72,3	73,7	73
	10 $\mu$ M	93,5	95,8	95	74,2	77,2	76
	7,5 $\mu$ M	97,6	98,1	98	80,9	82,2	82
	5 $\mu$ M	99,2	99,1	99	86	85,1	86
	2,5 $\mu$ M	102,7	103,4	103	94,4	94,7	95
	1,25 $\mu$ M	107,5	107,7	108	96,6	96	96
A1/3-Rd srmb	15 $\mu$ M	121,9	121,3	122	112,7	112,4	113
	12,5 $\mu$ M	117,2	118	118	108,1	107,8	108
	10 $\mu$ M	115,8	115,3	116	107,2	107,8	108
	7,5 $\mu$ M	114,6	113,6	114	107,6	106,6	107
	5 $\mu$ M	113,1	112,4	113	108,5	108,2	108
	2,5 $\mu$ M	111,9	111,9	112	105	104,2	105
	1,25 $\mu$ M	107,2	107,1	107	101,1	105,3	103

Table 4 One stage clotting activity of peptides A1/3, A1/3-Rd and A1/3-Rd-srmb (sequences see table 2). I2, buffer control.

Fig. 20 demonstrates the unchanged chromogenic activity of peptide A1/3-Rd. Peptides at a final concentration of 12 $\mu$ M or the buffer control (I2) were incubated in the presence of 2.3nM human FIXa (+). The chromogenic activity of peptide A1/3 and A1/3-Rd was found to be virtually unchanged and gave almost identical results in the chromogenic assay. The scrambled version of peptide A1/3, A1/5 as well as the buffer gave no significant FIXa

generation.

In the next series of experiments we set out to  
determine the individual role of any amino acid of the  
5 peptide core sequence by substituting each residue for  
the amino acid Alanine (Table 5).

Peptide	Sequence	Amino acid #	MW (D)	pI	Remark
A1/3	RRREGGGYYVNWYFDRRR (SEQ.ID.No. 18)	(18aa )	240 7	9, 9	Basic pI, soluble,
A1/3- 13	RRRAGGGYYVNWYFDRRR (SEQ.ID.No. 19)	(18aa )	234 9	10 .4	E <sub>1</sub> -A <sub>1</sub>
A1/3- 1	RRREAAGGYVNWYFDRRR (SEQ.ID.No. 20)	(18aa )	242 1	9. 9	G <sub>2</sub> -A <sub>2</sub>
A1/3- 2	RRREGAGGYVNWYFDRRR (SEQ.ID.No. 21)	(18aa )	242 1	9. 9	G <sub>3</sub> -A <sub>3</sub>
A1/3- 3	RRREGGAGYYVNWYFDRRR (SEQ.ID.No. 22)	(18aa )	242 1	9. 9	G <sub>4</sub> -A <sub>4</sub>
A1/3- 4	RRREGGGAYVNWYFDRRR (SEQ.ID.No. 23)	(18aa )	231 5	9. 9	Y <sub>5</sub> -A <sub>5</sub>
A1/3- 5	RRREGGGYAVNWYFDRRR (SEQ.ID.No. 24)	(18aa )	231 5	9. 9	Y <sub>6</sub> -A <sub>6</sub>
A1/3- 6	RRREGGGYYANWYFDRRR (SEQ.ID.No. 25)	(18aa )	237 9	9. 9	V <sub>7</sub> -A <sub>7</sub>
A1/3- 7	RRREGGGYYVAWYFDRRR (SEQ.ID.No. 26)	(18aa )	236 4	9. 9	N <sub>8</sub> -A <sub>8</sub>
A1/3- 8	RRREGGGYYVNAWYFDRRR (SEQ.ID.No. 27)	(18aa )	229 2	9. 9	W <sub>9</sub> -A <sub>9</sub>
A1/3- 9	RRREGGGYYVNWAFDRRR (SEQ.ID.No. 28)	(18aa )	231 5	9. 9	Y <sub>10</sub> -A <sub>10</sub>
A1/3- 10	RRREGGGYYVNWYADRRR (SEQ.ID.No. 29)	(18aa )	233 1	9. 9	F <sub>11</sub> -A <sub>11</sub>
A1/3- 11	RRREGGGYYVNWYFARRR (SEQ.ID.No. 30)	(18aa )	236 3	10 .5	D <sub>12</sub> -A <sub>12</sub>
A1/3- 12srm b	RRRYVYNGWGYFEGARRR (SEQ.ID.No. 31)	(18aa )	236 3	10 .4	Scrambled version

Table 5. Listed are the peptides designed to elucidate the role of any single amino acid within the peptide core sequence (E<sub>1</sub>G<sub>2</sub>G<sub>3</sub>G<sub>4</sub>Y<sub>5</sub>Y<sub>6</sub>V<sub>7</sub>N<sub>8</sub>W<sub>9</sub>Y<sub>10</sub>F<sub>11</sub>D<sub>12</sub>). The lower case numbers describe the position of the amino acid within the peptide. Alanine, an uncharged small amino acid, was substituted for each amino acid ("Alanine scan"). Also listed are the lengths of the peptides (amino acids #), the calculated molecular weights (MW, in Dalton (D) and the statistical isoelectric points (pI).

Each of the peptides was dissolved individually in imidazole buffer (50mM imidazole, 100mM NaCl, pH7.2) and subsequently diluted in clotting buffer (50mM imidazole, 100mM NaCl, 1% human albumin, pH7.4) to the desired final concentration. The peptides were analyzed for their chromogenic activity as well as for their potential to reduce the clotting time in a FVIII deficient plasma. The one-stage clotting assay was essentially done as described (see example 6). Clotting times (time from starting the reaction to the "clot"-formation were compared either against a buffer control or a control peptide (scrambled version).

Some of the results of the "Alanine scan" are given for the peptides A1/3-2 and A1/3-3. The change of G<sub>3</sub>-A<sub>3</sub> as exemplified in the peptide A1/3-2 yields high chromogenic activity and a strong reduction of the one-stage clotting time (34 seconds at a concentration of 12.5μM) in the presence of 2.2nM human FIXa. Peptide A1/3-3 (G<sub>4</sub>-A<sub>4</sub>) exhibits an optimum of chromogenic activity around a final concentration of 12μM with decreased activity at either higher or lower



concentrations. The peptide is somewhat inhibitory in a one-stage clotting assay at higher concentrations (12.5µM) in the absence of FIXa but becomes strongly active in the presence of 2.2nM FIXa (31 seconds, 12.5µM).

In the next series of experiments we set out to determine the individual role of any amino acid of the peptide core sequence by substituting each core residue for the amino acid glutamic acid (E) (see Table 6).

Peptide	Sequence	Amino-Acids	MW (D)	pI	Remark
A1/3	RRREGGGYYVNWYFDRRR	(18aa)	2407	9.9	Basic pI, soluble,
A1/3-22	RRREEGGGYYVNWYFDRRR (SEQ.ID.No. 32)	(18aa)	2479	9.5	G <sub>2</sub> -E <sub>2</sub>
A1/3-23	RRREGEGGYVNWYFDRRR (SEQ.ID.No. 33)	(18aa)	2479	9.5	G <sub>3</sub> -E <sub>3</sub>
A1/3-24	RRREGGEYYVNWYFDRRR (SEQ.ID.No. 34)	(18aa)	2479	9.5	G <sub>4</sub> -E <sub>4</sub>
A1/3-26	RRREGGGEYVNWYFDRRR (SEQ.ID.No. 35)	(18aa)	2373	9.4	Y <sub>5</sub> -E <sub>5</sub>
A1/3-27	RRREGGGYEVNWYFDRRR (SEQ.ID.No. 36)	(18aa)	2373	9.4	Y <sub>6</sub> -E <sub>6</sub>
A1/3-28	RRREGGGYYENWYFDRRR (SEQ.ID.No. 37)	(18aa)	2437	9.5	V <sub>7</sub> -E <sub>7</sub>
A1/3-29	RRREGGGYYVEWYFDRRR (SEQ.ID.No. 38)	(18aa)	2422	9.5	N <sub>8</sub> -E <sub>8</sub>
A1/3-30	RRREGGGYYVNEYFDRRR (SEQ.ID.No. 39)	(18aa)	2350	9.5	W <sub>9</sub> -E <sub>9</sub>
A1/3-31	RRREGGGYYVNWEFDRRR (SEQ.ID.No. 40)	(18aa)	2373	9.4	Y <sub>10</sub> -E <sub>10</sub>
A1/3-32	RRREGGGYYVNWYEDRRR (SEQ.ID.No. 41)	(18aa)	2389	9.5	F <sub>11</sub> -E <sub>11</sub>
A1/3-33	RRREGGGYYVNWYFERRR (SEQ.ID.No. 42)	(18aa)	2421	9.9	D <sub>12</sub> -E <sub>12</sub>
A1/3-34srmb	RRRGEYGEYWNGDFYRRR (SEQ.ID.No. 43)	(18aa)	2437	9.5	Scrambled version

Table 6. Listed are the peptides designed to elucidate

the role of any single amino acid within the peptide core sequence (E<sub>1</sub>G<sub>2</sub>G<sub>3</sub>G<sub>4</sub>Y<sub>5</sub>Y<sub>6</sub>V<sub>7</sub>N<sub>8</sub>W<sub>9</sub>Y<sub>10</sub>F<sub>11</sub>D<sub>12</sub>). The lower case numbers describe the position of the amino acid within the peptide. Glutamic acid, a negatively charged large amino acid, was substituted for each amino acid of the core sequence ("Glutamic acid scan"). Also listed are the lengths of the peptide (amino acids #), the calculated molecular weights (MW, in Dalton (D) and the statistical isoelectric points (pI).

10

Each of the peptides was solved individually in imidazole buffer (50mM imidazole, 100mM NaCl, pH7.2) and subsequently diluted in clotting buffer (50mM imidazole, 100mM NaCl, 1% human albumin, pH7.4) to the desired final concentration. The peptides derived from the "Glutamic acid scan" series were analyzed for their chromogenic FVIII-like activity as well as for their potential to reduce the clotting time in a FVIII deficient plasma. The one-stage clotting assay was essentially done as described (see example 6).

20

The peptide A1/3-24 showed some interesting properties. The molecule exhibited high chromogenic FVIII-like activity at concentrations between 6.5µM-12µM but lost activity at higher concentrations (up to 24µM). The peptide had no procoagulant activity in the absence of human FIXa but was strongly active in the presence of 2.2nM hFIXa.

25

In a second series of experiments we set out to improve the procoagulant activity of the antibody 198/B1 CDR3H derived peptide sequence B1. In a first step we improved the solubility of the original peptide sequence (B1; EGGGFTVNWYFDV) by removing the C-terminal Val residue

30

and adding several charged residues at the N- as well as the C-terminal end of the peptide. The resulting peptides B1/4, B1/6 (acidic pI), B1/7 (basic pI) and their scrambled versions B1/5, B1/7scr3 are readily  
 5 soluble in a variety of buffer systems at physiological pH.

Peptide	Sequence	Amino-acids	MW (D)	pI	Remark
B1	EGGGFTVNWYFDV (SEQ.ID.No. 44)	(13aa)	1491	6,0	Decreased solubility
B1/4	REGGGFTVNWYFDR (SEQ.ID.No. 45)	(14aa)	1704	7,9	Soluble,
B1/5	FGVGYRGETRNFWD (SEQ.ID.No. 46)	(14aa)	1704	8,0	Scrambled version, soluble
B1/6	EEEEGGGFTVNWYFDEEE (SEQ.ID.No. 47)	(18aa)	2166	5,0	Acidic pI soluble
B1/7	RRREGGGFTVNWYFDRRR (SEQ.ID.No. 48)	(18aa)	2329	9,9	Basic pI soluble
B1/7scr3	RRRFGVGYGETNFDWRRR (SEQ.ID.No. 49)	(18aa)	2329	9,9	Basic pI, soluble, scrambled version

Table 7 is a list of a series of antibody 198/B1 derived  
 10 peptides. Listed are the length of the peptide (aa, amino acids #), the calculated molecular weight (MW, in Dalton (D) and the statistical isoelectric point (pI).

Peptides B1/4 and B1/5 were soluble in 50mM Tris, 100mM  
 15 NaCl, pH=6.5. Both peptides were analyzed in a chromogenic FVIII assay. Peptide B1/4 but not the scrambled version B1/5 was found to have some chromogenic activity (data not shown).

20 Subsequently peptides B1/6, B1/7 and B1/7scr3 were analyzed. Each of the peptides was solved individually

in 50mM imidazole, 100mM NaCl, pH7.2 and subsequently diluted either in clotting buffer (50mM imidazole, 100mM NaCl; 1% human albumin, pH7.4) or in imidazole buffer to the desired final concentration. The peptides were  
5 analyzed for their chromogenic activity as well as for their potential to reduce the clotting time in a FVIII deficient plasma (table 8 & 9). The one stage clotting assay was essentially done as described (see example 6). Clotting times (time from starting the reaction to the  
10 "clot"-formation were compared either against a buffer control or a control peptide (scrambled version).

The FIXa activating activity (FVIII cofactor-like activity) from peptide B1/7 was first measured in the chromogenic assay described above.

15

As shown in Fig. 21, the addition of 2.4 $\mu$ M peptide B1/7 to the reaction mixture led to a well measurable generation of FXa. In contrast, the addition of 35 $\mu$ M Pefabloc Xa, a specific inhibitor of FXa protease  
20 activity, resulted in a significant reduction of the chromogenic substrate cleavage reaction (Fig. 22) thereby proving that there was indeed a peptide-FIXa mediated FXa generation. If there was no addition of FIXa and FX to the reaction mixture, no FXa was  
25 synthesized (Fig. 22). Peptide B1/6 and the control peptides B1/5 and B1/7scr3 exhibited no activity (data not shown).

Fig. 21 demonstrates the chromogenic activity of peptide B1/7. The peptide at a final concentration of 2.4 $\mu$ M or  
30 the buffer control (IZ) were incubated in the presence of 2.3nM human FIXa.

In Fig. 22 peptide B1/7 at a final concentration of 2.4 $\mu$ M or the buffer control (IZ) were incubated in the

presence of 2.3nM human FIXa (as indicated either as "+2.3nM hFIXa" or "+"). The chromogenic activity of peptide B1/7 was found to be dependent on the presence of FIXa and FX since no reaction is detectable when FIXa and FX are left out of the reaction (w/o FIXa/FX). To prove that the peptide B1/7 mediates indeed FXa generation, the FXa specific protease inhibitor Pefabloc Xa was added to the reaction mix (35µM Pefabloc Xa). In a second set of experiments, the procoagulant effect of peptides B1/6, B1/7 and B1/7scr3 were tested in a aPTT based one-step coagulation assay. The experiments were done essentially as described in Example 6. The results are shown in tables 8 and 9.

Peptide	12,5µM (-)	1.25µM (-)	0.125µM (-)	12,5nM (-)	Buffer (-)	remarks
B1/6	115	110	111	111	110	
B1/7	157	112	109	110	110	
B1/7scr3	115	105	106	105	107	

Table 8: FVIII deficient plasma was incubated either with peptides B1/6, B1/7scr3 or B1/7 in the absence of activated human FIX. As a negative control, plain buffer was added to the deficient plasma. The clotting times for the various combinations are given. Under these conditions, peptide B1/7 at its highest concentration (12.5µM) becomes inhibitory to the coagulation process as indicated by the extended clotting time of 157 seconds.

Peptide	12,5µM (+)	1.25µM (+)	0.125µM (+)	12,5nM (+)	Buffer (+)	remarks
B1/6	103	100	101	100	100	
B1/7	83	92	99	99	100	
B1/7scr3	102	94	94	94	94	

Table 9: FVIII deficient plasma was incubated either with peptides B1/6, B1/7scr3 or B1/7 in the presence of activated human FIX. As a negative control, plain buffer was added to the deficient plasma. The clotting times for the various combinations are given. In the presence of FIXa, peptide B1/7 becomes procoagulant as indicated by the reduced clotting time (83 seconds compared to 102 seconds for the scrambled peptide and 100 seconds for the buffer control)

**Example 12: Procoagulant activity of peptide derivatives obtained from CDR3 regions of anti- FIX/FIXa-antibodies in FVIII inhibitor plasma**

To assay for the procoagulant activity of peptide A1/3 in FVIII inhibitor plasma the following experiment was carried out. We performed a standard aPTT based one stage clotting assay, but instead of FVIII deficient plasma we employed FVIII inhibitor plasma. The inhibitory potency of the plasma was 8.1 Bethesda Units per ml.

		w/o FIXa	w/o FIXa			FIXa	FIXa	
	Peptide conc.	sec	sec	Average sec		sec	sec	average sec
IZ	0	104,8	103,6	104		94,2	94,1	94
A1/3	12,5µM	85,8	85,3	86		61	60,2	61
	10µM	88,4	87,9	88		61,3	61,8	62
	7,5µM	93,7	92,7	93		68,8	70,9	70
	5µM	101,5	101,1	101		81	82	82
	2,5µM	106,1	105,3	106		90,2	90,5	90
	1,25µM	104,5	104,3	104		91,3	91,4	91

Table 10: Various amounts of peptide A1/3 (12.5µM-1.25µM) were added to FVIII inhibitor plasma (either in the presence (FIXa) of 2.2nM FIXa or in the absence (w/o FIXa). As a negative control, plain buffer was added to the plasma (IZ). Experiments were done in duplicate and the average (aver.) was calculated. The clotting times (in seconds) for the various combinations are given. It is easily appreciable that the peptide A1/3 reduces (in a dose dependent manner) the clotting time of FVIII inhibitor plasma in the presence of FIXa but, although albeit to a much lesser extent, also in the absence of

FIXa.

**Example 13: Conversion of the 196/C4 IgM into IgG1**

Since some IgM antibodies demonstrate high FVIII-like activity in chromogenic assays, attempts were made to convert such IgM antibodies into IgG antibodies (though antibody derivatives such as Fab, F(ab)<sub>2</sub>, scFv, etc. could also be produced). Described in detail below is the rescue of the IgM variable region genes.

Expression vector pBax-IgG1 (Fig. 23) was first constructed from vectors pSI (Promega) and pEF/Bsd (Invitrogen) through multiple cloning steps. B-lymphocytes of a donor are purified from blood and mature mRNA purified from these cells using the "micro-mRNA purification-kit" (Pharmacia). The cDNA of a human kappa chain and a human gamma 1 chain are prepared employing the "you-primefirst-strand-cDNA-kit" (Pharmacia) using specific primers.

The coding sequence of a human kappa light chain constant domain is amplified from the cDNA by PCR using specific primers.

The gene of a human gamma 1 chain constant region (CH1-hinge-CH2-CH3) is amplified from the cDNA by PCR using specific primers.

The PCR product of the light chain constant domain is digested with XbaI and NheI and inserted into digested pSI. The resultant vector is cleaved with EcoRI and XbaI and annealed oligonucleotides are inserted, resulting in vector pSI-Ckappa. The annealed oligonucleotides provide for the leader and the SacI-XbaI sites for insertion of the kappa chain variable region. The PCR product of the human gamma 1 chain constant region is digested with SpeI and BamHI and inserted into digested pSI. The resultant vector is



cleaved with SpeI and NotI and annealed oligonucleotides are inserted resulting in vector pSI-Cgamma. The annealed oligonucleotides provide for the leader and the XhoI-BstEI sites for insertion of the heavy chain variable region. Vector pEF/Bsd is digested NheI and SfiI, blunt ended by Klenow treatment and the whole expression cassette of pSI-Ckappa, excised with BglII and BamHI, is inserted (after Klenow treatment). The resultant vector is digested with EcoRI and HindIII and treated with Klenow. The whole expression cassette of pSI-Cgamma is excised with BglII and BamHI and is inserted (after Klenow treatment). The resultant vector is named pBax-IgG1.

The light chain variable region can be inserted in between the SacI-XbaI sites, yielding the complete coding-sequence of a kappa light chain. The heavy chain variable region can be cloned in between the XhoI-BstEI sites, resulting in a complete IgG1 heavy chain gene. Both open reading frames are expressed under the control of the SV40-promoter and harbour the coding sequence of a signal peptide at the 5' end of the genes for secretion of the heavy and light chains into the endoplasmatic reticulum. Transfection into COS cells allows the expression of an IgG1 with the same binding properties as the parental IgM.

Construction of the plasmid pBax-196/C4 is further accomplished by amplifying the VH of the 196/C4 scFv (subcloned as described in Experiment 10) by PCR using specific primers. The PCR product is digested with XhoI and BstEII and inserted into XhoI and BstEII digested pBax IgG1. The VL of the 196/C4 scFv is amplified by PCR using specific primers. The PCR product is digested with SacI and XbaI and inserted into SacI and XbaI-digested

pBax IgG1-VH. The resultant vector (pBax-196/C4) is transfected into COS cells by electroporation, and hybrid IgG1 molecules (murine variable region and human constant region) with the same specificity as the parental IgM is expressed.

**Example 14: Activation of FIXa amyolytic activity by anti-FIXa antibodies:**

Briefly, 20µl factor IXa (containing 200mU FIXa (Stago)) were incubated at 37°C, with 200µl of reaction buffer (50mM TrisHCl pH7.4, 100mM NaCl, 5mM CaCl<sub>2</sub> and 40% Ethyleneglycol), 25µl of FIXa substrate (CH<sub>3</sub>SO<sub>2</sub>-D-CHG-Gly-Arg-pNA, AcOH, 10µM/ml, Pentapharm LTD) in the absence or presence of various amounts of anti-FIX antibodies 198/B1 (IgG isotype) or 196/AF1 (IgM isotype). Specific cleavage of FIXa substrate was monitored at 405nm in an ELISA reader.

The presence of the anti-FIX antibodies enhanced the amyolytic activity of FIXa at least 2 fold.

Fig. 24 shows the increase of the amidolytic activity of FIXa in the presence of antibody 198/B1 (Fig. 24A) and antibody 198/AF1 (Fig. 24B).

**Example 15: FVIII-like activity exhibited by Fab fragments derived from anti-FIX/FIXa-antibodies.**

Fab fragments of anti-FIX/FIXa antibodies were prepared and purified according to standard protocols. Briefly, 1ml antibody 198/A1 (4mg/ml in 50mM imidazole, 100mM NaCl, pH7.4) was incubated overnight with 87µl fragmentation buffer (1M Na Acetate, 10mM EDTA 67.5mg/ml L-cysteine) and 0.25mg papain (immobilized on agarose beads), at 37°C. The preparation was filtered to remove the papain. L-histidine was added (final concentration 50mM) and afterwards the pH was adjusted to 7.0. Finally, solid NaCl is added to give a final

concentration of 1M.

Subsequently, the 198/A1 Fab fragment was purified by binding to protein L: We used ImmunoPure Immobilized PROTEIN L Plus (Pierce) in a PHARMACIA XK 16/20 Column  
 5 (gel-volume: 2ml) Buffers for chromatography were: 1) equilibration-buffer : 50mM L-histidine pH 7.0; 1M NaCl; 0,1% (w/v) NaN<sub>3</sub>; 2) wash-buffer: 50mM L-Histidine pH 7.0; 0.1% (w/v) NaN<sub>3</sub>; 3) elution-buffer: 100 mM glycine pH 2.5; 0.1% (w/v) NaN<sub>3</sub>; and 4) neutralization  
 10 buffer: 2M Tris/Cl pH 8,0;

Chromatography was essentially done by following steps 1 to 7 described in table 11. In order to neutralize the low pH of the elution buffer "Fraction-tubes" were pre-  
 15 loaded with 0.2 ml 2M Tris pH 8.0.

	STEP	BUFFER	Flow rate	Vol.	CV	Fractions
1.	column-wash	elution-buffer	2,0 ml/min	10 ml	5	waste
2.	equilibratio n	equi- buffer	2,0 ml/min	10 ml	5	waste
3.	sample-load	sample	1,0 ml/min	x ml	x	flow-through
4.	wash 1	equi- buffer	1,0 ml/min	20 ml	10	flow-through
5.	wash 2	wash- buffer	1,0 ml/min	10 ml	5	flow-through
6.	elution	elution- buffer	1,0 ml/min	15 ml	7,5	1,0 ml fractions-
7.	neutralizati on	wash- buffer	2,0 ml/min	10 ml	5	waste

Table 11

The final 198/A1 Fab preparation was dialyzed against  
 20 50mM imidazole, 100mM NaCl, pH7.4 and analyzed in a chromogenic FVIII assay as described above (Fig. 25). Compared to an intact antibody, the 198/A1 Fab fragment

has somewhat less activity; however, the Fab fragment still gives rise to FIX dependent FXa generation.

Fig. 25 demonstrates the chromogenic FVIII-like activity of the antibody 198/A1 Fab fragment in the presence of 2.3nM human FIXa. As a positive control we used the intact antibody 198/A1 as well as 7.5pM FVIII. Buffer control (IZ) instead of 198/A1 Fab fragment or FVIII was used as a negative control.

**Example 16: FVIII-like activity exhibited by fusion proteins between scFv fragments of anti-FIX/FIXa antibodies and E. coli alkaline phosphatase.**

The single chain Fv fragment (see example 10) of antibody 198/B1 (subclone AB2) was fused to the N-terminus of E. coli alkaline phosphatase employing the pDAP2 vector system (Kerschbaumer et al., 1996). Two identical clones were isolated and designated pDAP2-198AB2#1 and pDAP2-198AB2#100 (Fig. 26). The resulting fusion proteins were expressed in E. coli, purified by metal affinity chromatography (Kerschbaumer et al., 1997) and analysed in a standard chromogenic assay (Fig. 27).

Fig. 27 demonstrates the chromogenic FVIII-like activity of two antibody 198/B1 (subclone AB2) scFv fragment-alkaline phosphatase fusion proteins (198AB2#1 and 198AB2#100) in the presence of 2.3nM human FIXa. As a positive control we used 7.5pM FVIII.

**Example 17: FVIII-like activity exhibited by a bivalent miniantibody.**

In order to obtain a bivalent miniantibody, the scFv fragment of antibody 198/B1 (subclone AB2) was fused to an amphipathic helical structure employing the pZip1 vector system (Kerschbaumer et al. (*Analytical Biochemistry* 249, 219-227, 1997)). Briefly, the gene of

the 198/B1 scFv fragment was isolated from the plasmid pDAP-198AB2#100 (example 16) by digestion with SfiI and NotI. The DNA fragment was gel purified and inserted in the SfiI/NotI digested vector pZip1. The resulting  
5 plasmid was sequenced and designated pZip-198AB2#102 (Fig.28). In parallel, we constructed a miniantibody version from an irrelevant monoclonal antibody termed #8860. In a first step, the single chain Fv fragment of antibody #8860 was assembled in the vector pDAP2. The  
10 cloning was done essentially as described in example 10. The construct was named pDAP2-8860scFv#11 (Fig. 29). Subcloning of the scFv fragment contained within pDAP2-8860scFv#11 into plasmid pZip1 (see above) yielded the miniantibody construct p8860-Zip#1.2 (Fig. 30). Since  
15 antibody #8860 does not react with FIX/FIXa (as judged by Western Blot and ELISA analysis) it represents an appropriate negative control. Subsequently, the miniantibody proteins were expressed in E. coli and purified from bacterial supernatants by binding to  
20 Protein L according to the following protocol:  
For affinity chromatography we used ImmunoPure Immobilized PROTEIN L Plus (Pierce) in a PHARMACIA XK 16/20 Columns having a gel-volume of 4ml  
Buffers employed were: 1) equilibration-buffer : 50mM  
25 L-Histidine pH 7.0, 1M NaCl, 0.1% (w/v) NaN<sub>3</sub>, wash-buffer: 50mM L-histidine pH 7.0, 0.1% (w/v) NaN<sub>3</sub>,  
elution-buffer: 100 mM glycine pH 2.5, 0.1% (w/v) NaN<sub>3</sub>,  
and neutralization buffer: 2M Tris/Cl pH 8.0  
30 Samples were prepared as follows: The bacterial culture supernatant was obtained by centrifugation of the bacterial expression culture (11,000 x g, 4°C, 10 minutes). 470 g of ammonium-sulphate was added to 1

liter of supernatant and the solution stirred on ice for 1 hour to precipitate the protein. The precipitate was pelleted at 14,000 x g for 35 minutes at 2°C and re-dissolved in 100 ml 20mM Tris pH 7.0. Subsequently the concentrate was dialyzed against 20mM Tris pH 7.0, L-histidine was added to a final concentration of 50mM and the pH was adjusted to 7.0. Finally, solid NaCl was added to give a final concentrations of 1M. Before loading on the column, a sample was first centrifuged at 16,000 x g for 15 min at room temperature and then filtered through a 0.45µm sterile filter.

Chromatography was essentially done by following steps 1 to 7 described in table 12. In order to neutralize the low pH of the elution buffer "Fraction-tubes" were pre-loaded with 0.2 ml 2M Tris pH 8.0.

	STEP	BUFFER	Flow rate	Vol.	CV	Fractions
1.	column-wash	elution-buffer	2.0 ml/min	20 ml	5	waste
2.	equilibrati on	equi-buffer	2.0 ml/min	20 ml	5	waste
3.	sample-load	sample	1.0 ml/min	x ml	x	flow-through
4.	wash 1	equi-buffer	1.0 ml/min	40 ml	10	flow-through
5.	wash 2	wash-buffer	1.0 ml/min	20 ml	5	flow-through
6.	elution	elution-buffer	1.0 ml/min	30 ml	7.5	1,0 ml fractions-
7.	neutralizat ion	wash-buffer	2.0 ml/min	20 ml	5	waste

Table 12. The final 198/B1 (subclone AB2) miniantibody preparation (designated 198AB-Zip#102) and the negative control 8860-Zip#1.2 were dialyzed against 50mM imidazole, 100mM NaCl, pH7.4 and analyzed in a

chromogenic FVIII assay as described above (Fig. 31).

As can be seen in Fig. 31, the miniantibody construct 198AB-Zip#102 gives rise to substantial FXa generation (compare to FVIII) whereas the negative control miniantibody 8860-Zip#1.2 does not.

Fig. 31 demonstrates the chromogenic FVIII-like activity of the 198/B1 (subclone AB2) miniantibody 198AB-Zip#102 in the presence of 2.3nM human FIXa. As a positive control we used 4.8pM FVIII whereas an unrelated miniantibody (8860-Zip#1.2) and plain reaction buffer (IZ) served as negative controls.

**Example 18: FVIII-like activity exhibited by anti-FIXa/FIX antibody scFv fragments**

The single chain Fv fragment of antibody 198/B1 (subclone AB2) as well as the scFv fragment of antibody #8860 were expressed employing the pMyHis6 vector system. Vector pMyHis6 (Fig. 32 & 33) was constructed by cleaving vector pCOCK (Engelhardt et al., 1994, Biotechniques, 17:44-46) with NotI and EcoRI and insertion of the following oligonucleotides: mychis6-co: 5'ggccgcagaacaaaaactcatctcagaagaggatct gaatggggcgccacatcaccatcaccatcactaataag 3' (SEQ.ID.NO. 79) and mycchis-

ic:5'aattcttattagtgatggtgatggtgatgtgccgccccattcagatcctct tctgagatgagtttttgttctgc 3' (SEQ.ID.NO. 80)

Fig. 32 shows a schematic representation of the plasmid pMyHis6. The c-myc-tag sequence is used to detect the scFv fragment in an ELISA or a Western Blot analysis (Evan et al., Mol.Cell.Biol., 1985, 5(12), pp. 3610-6). The His6-tag sequence was included to facilitate the purification of scFv fragments by metal ion chromatography (Hochuli et al., 1988. Biotechnology,

6:1321-1325). The plasmid contains the lacZ gene promoter (PlacZ) the PelB-leader sequence (see legend Fig. 26) an E. coli origin of replication (colE1ori) and a M13 phage origin of replication (M13ori). To allow for specific selection, the plasmid also carries the gene for the enzyme  $\beta$ -lactamase (AmpR) mediating resistance against the antibiotic ampicillin.

The gene of the 198/B1 (clone AB2)-scFv was rescued from plasmid pDAP2-198AB2#100 (example 16) by digestion with SfiI and NotI and inserted into SfiI/NotI cleaved pMycHis6. The resultant plasmid was designated pMycHis-198AB2#102. Fig. 34 shows the nucleotide and amino acid sequence of 198AB2 scFv (linked to the c-myc-tag and the His6- tag):the resulting ORF of the expression vector is named pMycHis6-198AB2#102. Vector pMycHis6 was constructed by cleaving vector pCOCK (Engelhardt O. et al, BioTechniques 17, 44-46, 1994) NotI - EcoRI and inserting the following annealed oligonucleotides:  
(5'-GGCCGCAGAACAAAACTCATCTCAGAAGAGGATCTGAATGGG  
GCGGCACATCACCATCACCATCACTAATAAG - 3' (SEQ.ID.No. 103)  
and 5'- TTATTAGTGATGGTGATGGT  
GATGTGCCGCCCCATTTCAGATCCTCTTCTGAGATGAGTTTTTGTCTGTC-  
3' (SEQ.ID.NO. 104)). The resultant vector, named pMycHis6, was cleaved SfiI - NotI and the gene of scFv 198AB2 was swapped into this vector from vector pDAP2-198AB2#100.

In analogy to the 198AB2 construct, the #8860 scFv fragment was cloned from a plasmid designated pDAP2-8860scFv clone 11. The pure scFv protein of #8860 was designated 8860-M/H#4c (plasmid p8860-M/H#4c, Fig. 35). The scFv proteins were expressed in E. coli and affinity purified from bacterial supernatants on Protein L columns (see example 17). The final MycHis-198AB2#102



and 8860-M/H#4c preparations were dialyzed against 50mM imidazole, 100mM NaCl, pH7.4 and analyzed in a chromogenic FVIII assay as described above (Fig. 36).

- 5 As can be seen in Fig. 36, the scFv construct MycHis-198AB2#102 gave rise to a substantial FXa generation whereas the negative controls 8860-M/H#4c and plain reaction buffer (IZ) did not.

Fig. 36 demonstrates the chromogenic FVIII-like activity  
10 of the 198/B1 (subclone AB2) scFv fragment (MycHis-198AB2#102) in the presence of 2.3nM human FIXa. As a positive control we used 4.8pM FVIII whereas a unrelated scFv (8860-M/H#4c) and plain reaction buffer (IZ) served as negative controls.

15

Claims:

1. An antibody or antibody derivative against factor  
5 IX/factor IXa which increases the procoagulant activity  
of FIXa.
2. An antibody or antibody derivative according to  
claim 1, wherein said antibody or antibody derivative  
10 increases the procoagulant activity of FIXa in the  
presence of FVIII inhibitors.
3. An antibody according to any one of claim 1 wherein  
said antibody is selected from the group consisting of  
15 IgG, IgM, IgA and IgE antibodies.
4. An antibody or antibody derivative according to  
claim 1, wherein said antibody or antibody derivative is  
selected from the group consisting of monoclonal  
20 antibodies, antibody fragments, chimeric antibodies,  
humanized antibodies, single chain antibodies,  
bispecific antibodies, diabodies, and di-, oligo- or  
multimers thereof.
- 25 5. An antibody derivative according to claim 1,  
wherein said antibody derivative comprises a complement  
determining region (CDR) peptide.
6. An antibody derivative according to claim 5,  
30 wherein said CDR peptide is a CDR3 peptide.

7. An antibody derivative according to claim 6,  
wherein said CDR3 peptide comprises an amino acid  
sequence selected from the group consisting of:  
Tyr-Gly-Asn-Ser-Pro-Lys-Gly-Phe-Ala-Tyr;  
5 Cys-X-X-Tyr-Gly-Asn-Ser-Pro-Lys-Gly-Phe-Ala-Tyr-X-X-Cys,  
wherein  
X may be any desired amino acid;  
Tyr-Gly-Asn-Ser-Pro-Lys-Gly-Phe-Ala-Tyr;  
Asp-Gly-Gly-His-Gly-Tyr-Gly-Ser-Ser-Phe-Asp-Tyr; and  
10 Phe-Arg-Asn-Arg-Gly-Met-Thr-Ala-Leu-Leu-Lys-Val-Ser-Ser-  
Cys-Asp.
8. An antibody or antibody derivative according to  
claim 1, wherein the variable region of said antibody or  
15 antibody derivative comprises amino acids 1 to 357  
and/or amino acids 403 to 726 according to Fig. 14.
9. An antibody or antibody derivative according to  
claim 8, wherein said antibody or antibody derivative  
20 additionally comprises an artificial linker sequence.
10. An antibody or antibody derivative according to  
claim 1, wherein the variable region of said antibody or  
antibody derivative comprises amino acids 1 to 363  
25 and/or amino acids 409 to 747 according to Fig. 15.
11. An antibody or antibody derivative according to  
claim 10, wherein said antibody or antibody derivative  
additionally comprises an artificial linker sequence.  
30
12. An antibody or antibody derivative according to  
claim 1, wherein the variable region of said antibody

or antibody derivative comprises amino acids 1 to 366 and/or amino acids 412 to 747 according to Fig. 16.

13. An antibody or antibody derivative according to claim 12, wherein said antibody or antibody derivative additionally comprises an artificial linker sequence.

14. A hybridoma cell line expressing an antibody or antibody derivative against factor IX/factor IXa according to claim 1.

15. A hybridoma cell line according to claim 14, wherein said cell line is selected from the group consisting of #196/AF1, #196/AF2, #193/AD3, #193/K2-1, #198/AC1/1, #198/AM1, #198/A1, #198/B1, #198/AP1, 198/A1, 198/B1, 198/BB1, 198/A1, 198/B1, 198/BB1.

16. An antibody or antibody derivative according to claim 1, which is expressed by a hybridoma cell line according to claim 14.

17. A DNA molecule, wherein said DNA molecule encodes an antibody or an antibody derivative according to claim 1.

25

18. A pharmaceutical preparation comprising an antibody or antibody derivative according to claim 1 and a pharmaceutically acceptable carrier.

19. A preparation according to claim 18, additionally comprising factor IX $\alpha$  and/or factor IX $\beta$ .

30

20. A method for treating patients afflicted with blood coagulation disorders comprising administering a pharmaceutically effective amount of the preparation of claim 18 to said patients.

5

21. The method of claim 20, wherein said blood coagulation disorders are selected from the group comprising hemophilia A and hemorrhagic diathesis.

10 22. The method of claim 21, additionally comprising the step of selecting hemophilia inhibitor patients.

23. A method of obtaining an antibody or antibody derivative which interacts with factor IX/factor IXa and increases the procoagulant activity of Factor IXa, comprising the steps of:

15 - immunizing an immunocompetent mouse with an antigen selected from the group consisting of FIX, FIX $\alpha$ , FIX $\alpha$  $\beta$  or fragments thereof,

20 - isolating spleen cells of the immunized mouse,

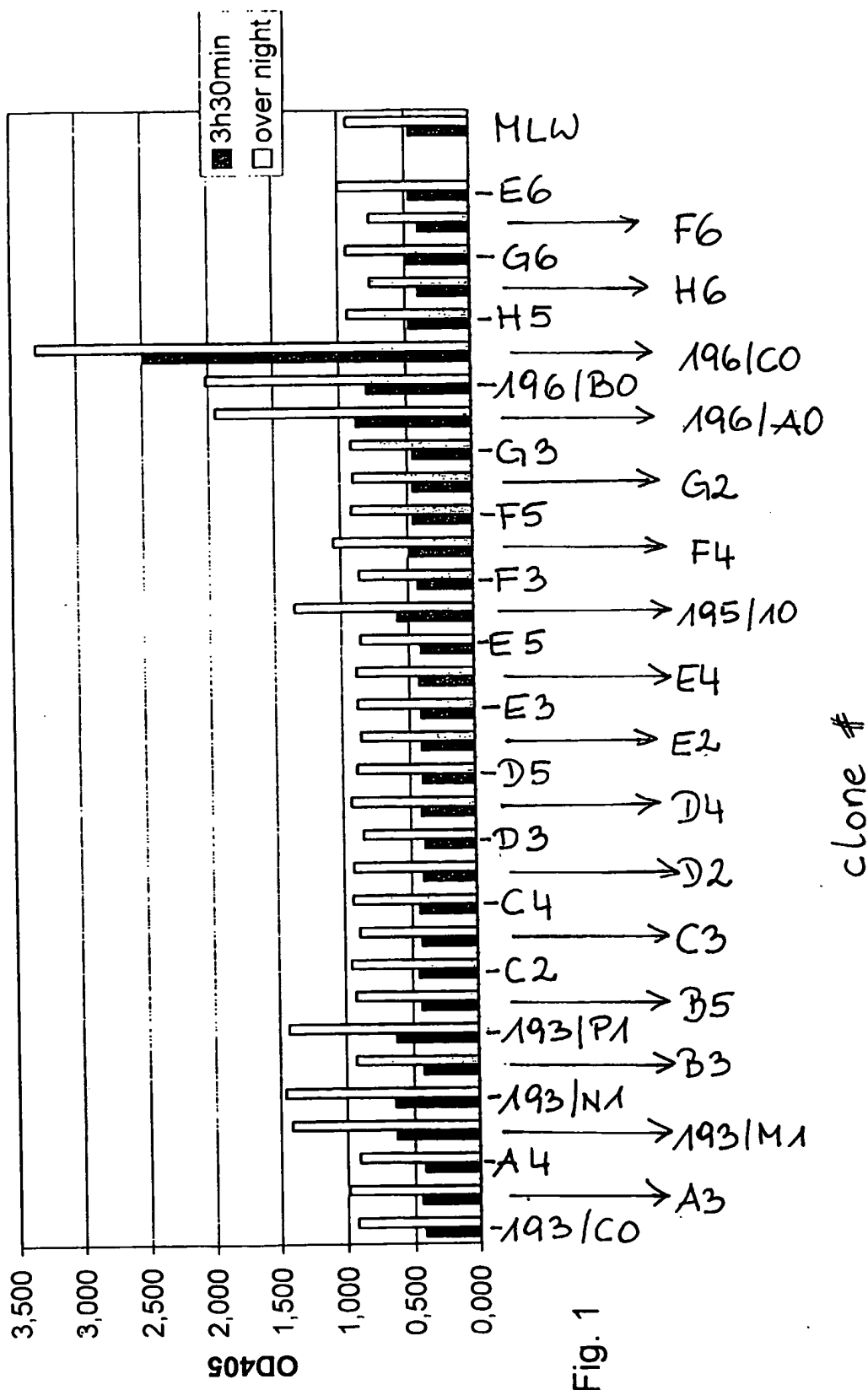
- producing hybridoma clones,

- screening the hybridoma cell supernatants for an increase in the procoagulant activity of Factor IXa, isolating and purifying the antibodies or antibody

25 derivatives from hybridoma cell supernatants which exhibit an increase in the procoagulant activity of factor IXa.

24. Use of an antibody or antibody derivative according to claim 1 for increasing the amidolytic activity of

30 factor IXa.

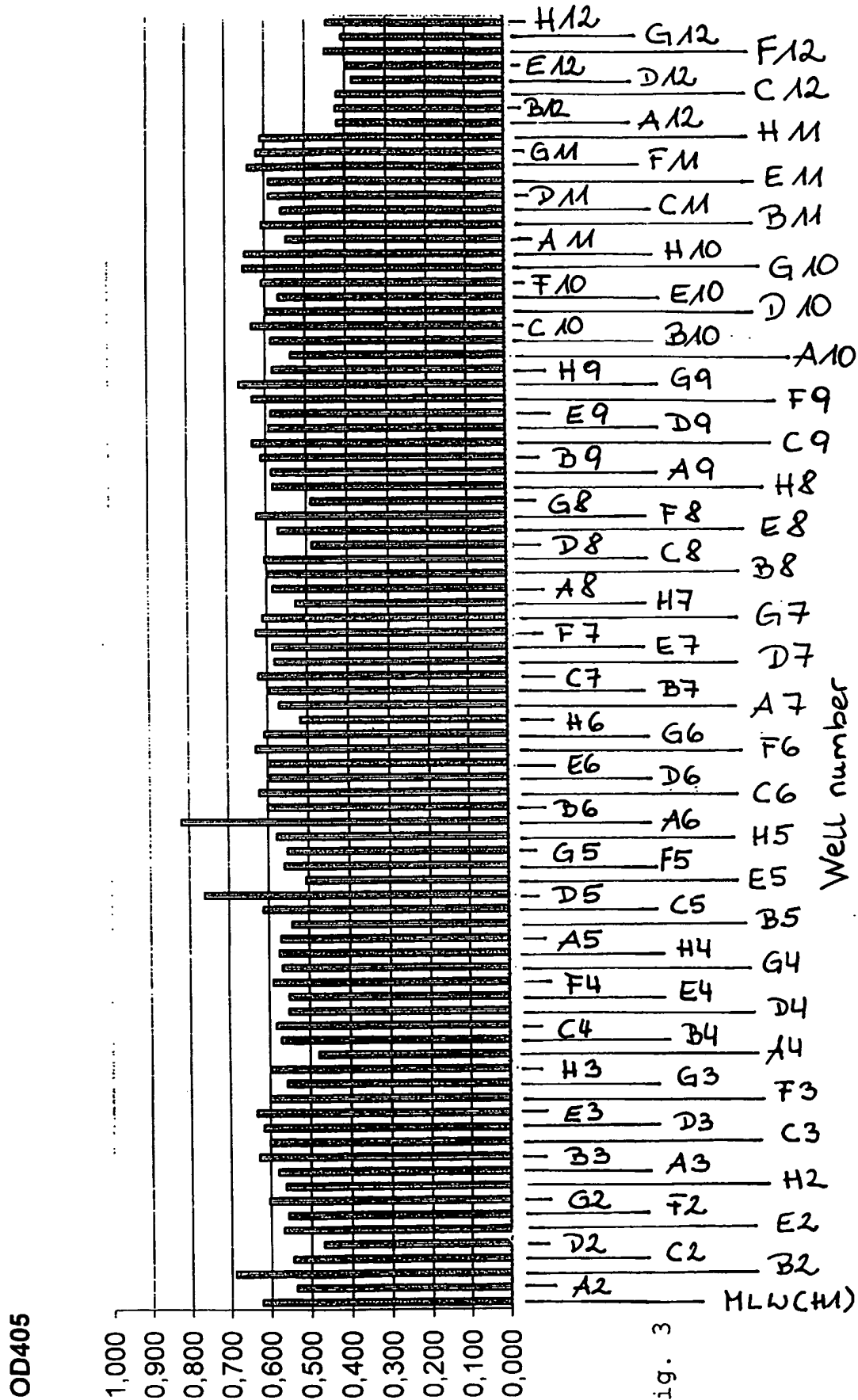


OD405

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	E 12	D 12	C 12
	B 12	A 12	H 11
	G 11	F 11	E 11
	D 11	C 11	B 11
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	F 10	E 10	D 10
	C 10	B 10	A 10
	H 9	G 9	F 9
	E 9	D 9	C 9
	B 9	A 9	H 8
	G 8	F 8	E 8
	D 8	C 8	B 8
	A 8	H 7	G 7
	F 7	E 7	D 7
	C 7	B 7	A 7
	H 6	G 6	F 6
	E 6	D 6	C 6
	B 6	A 6	H 5
	G 5	F 5	E 5
	D 5	C 5	B 5
	A 5	H 4	G 4
	F 4	E 4	D 4
	C 4	B 4	A 4
	H 3	G 3	F 3
	E 3	D 3	C 3
	B 3	A 3	H 2
	G 2	F 2	E 2
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	A 2	H 1	G 1
	F 1	E 1	D 1
	C 1	B 1	A 1

fig 2

Well number





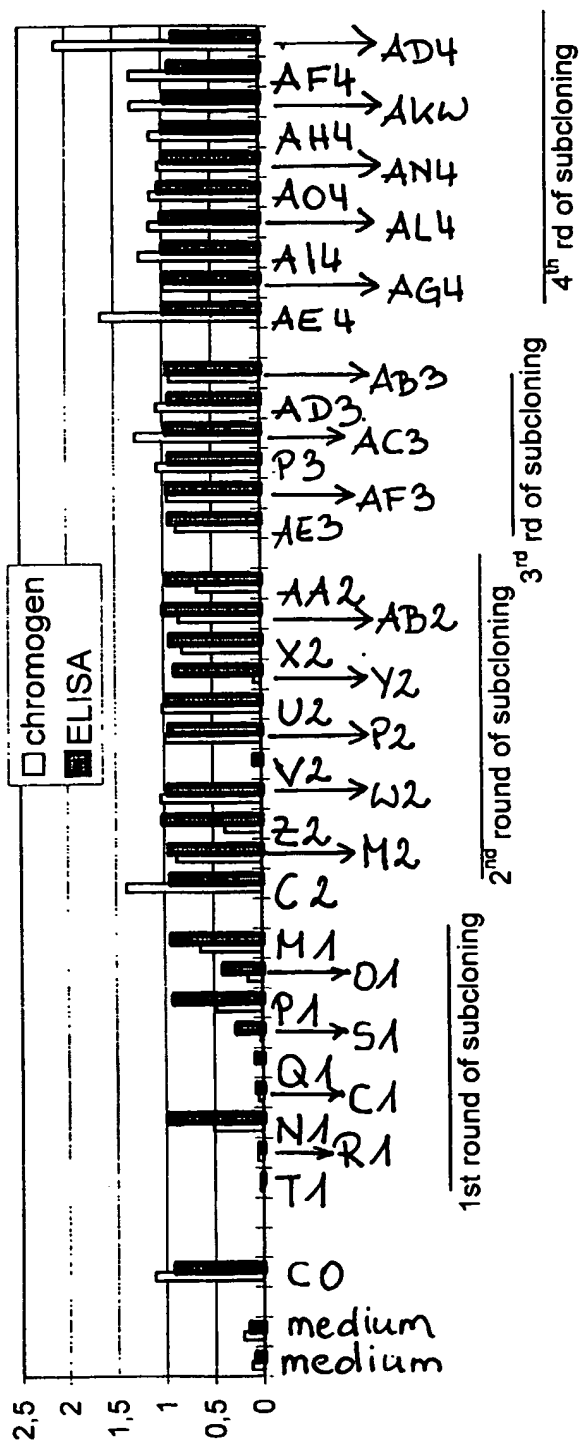


Fig. 4

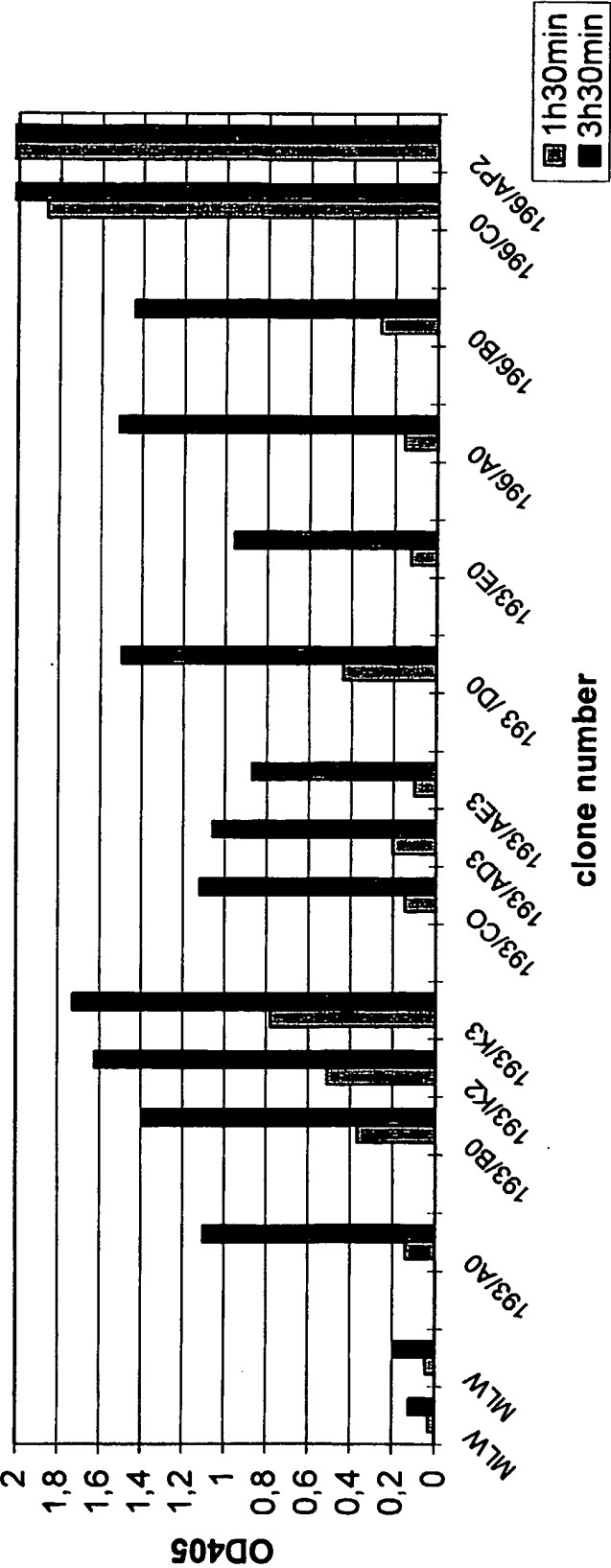


Fig. 5

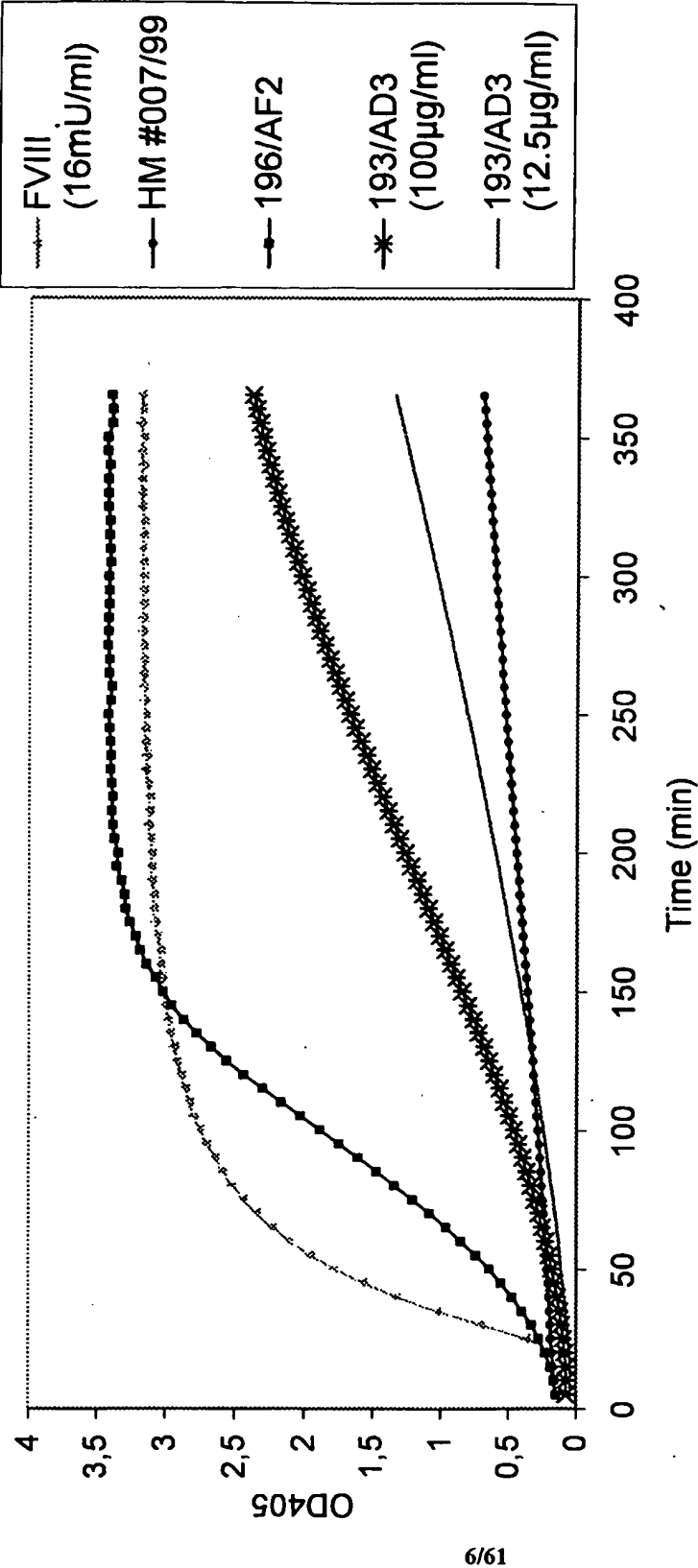


Fig. 6A

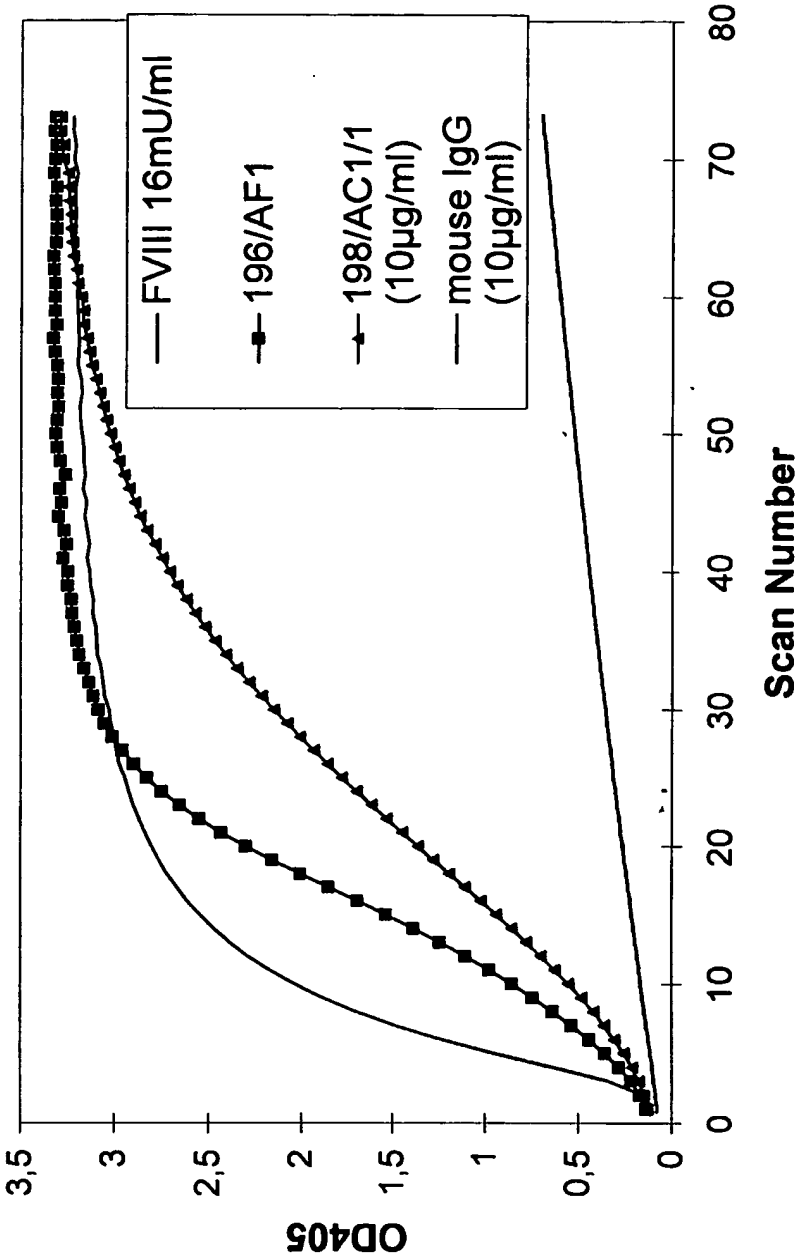


Fig. 6B

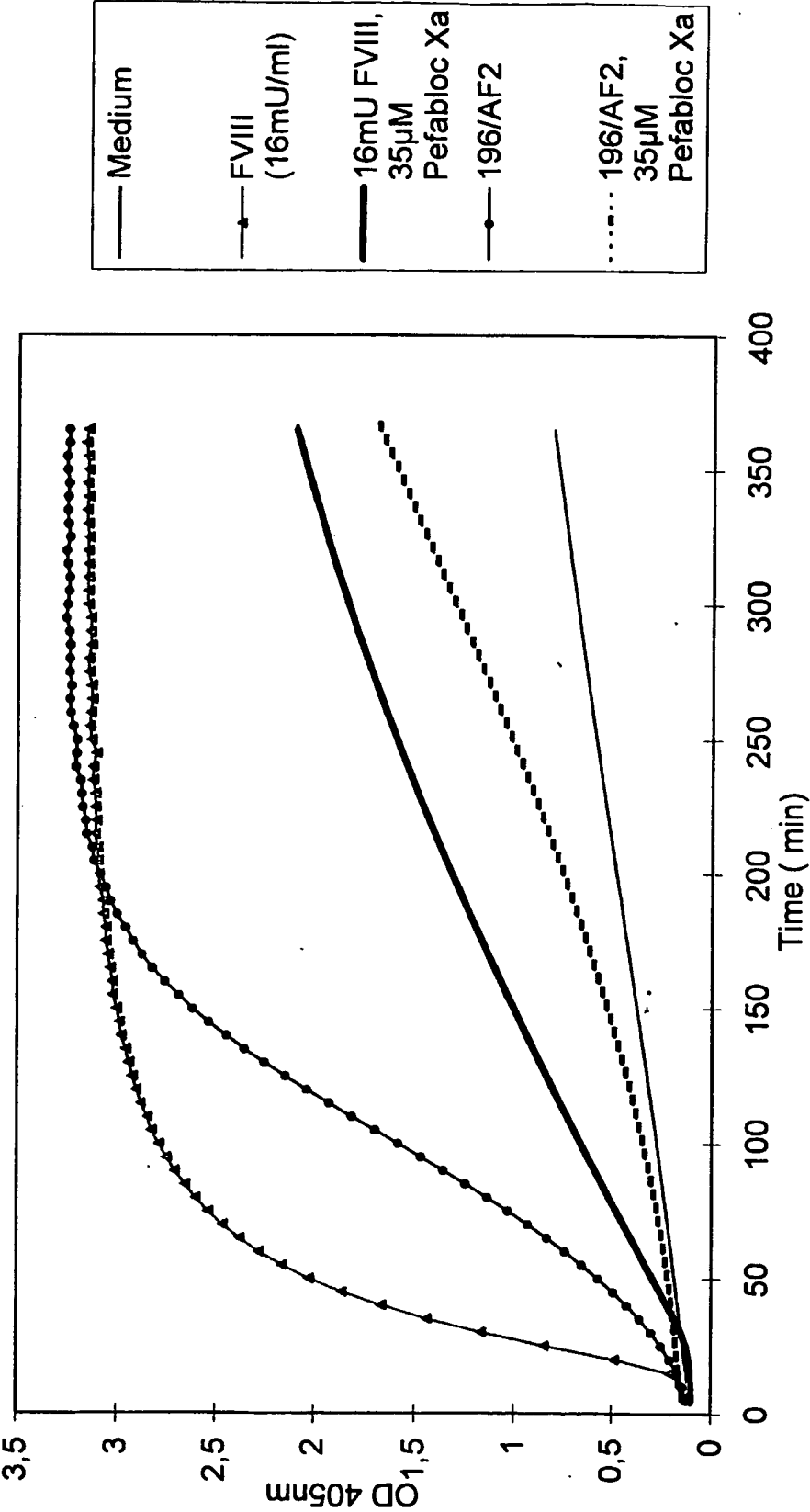


Fig. 7A

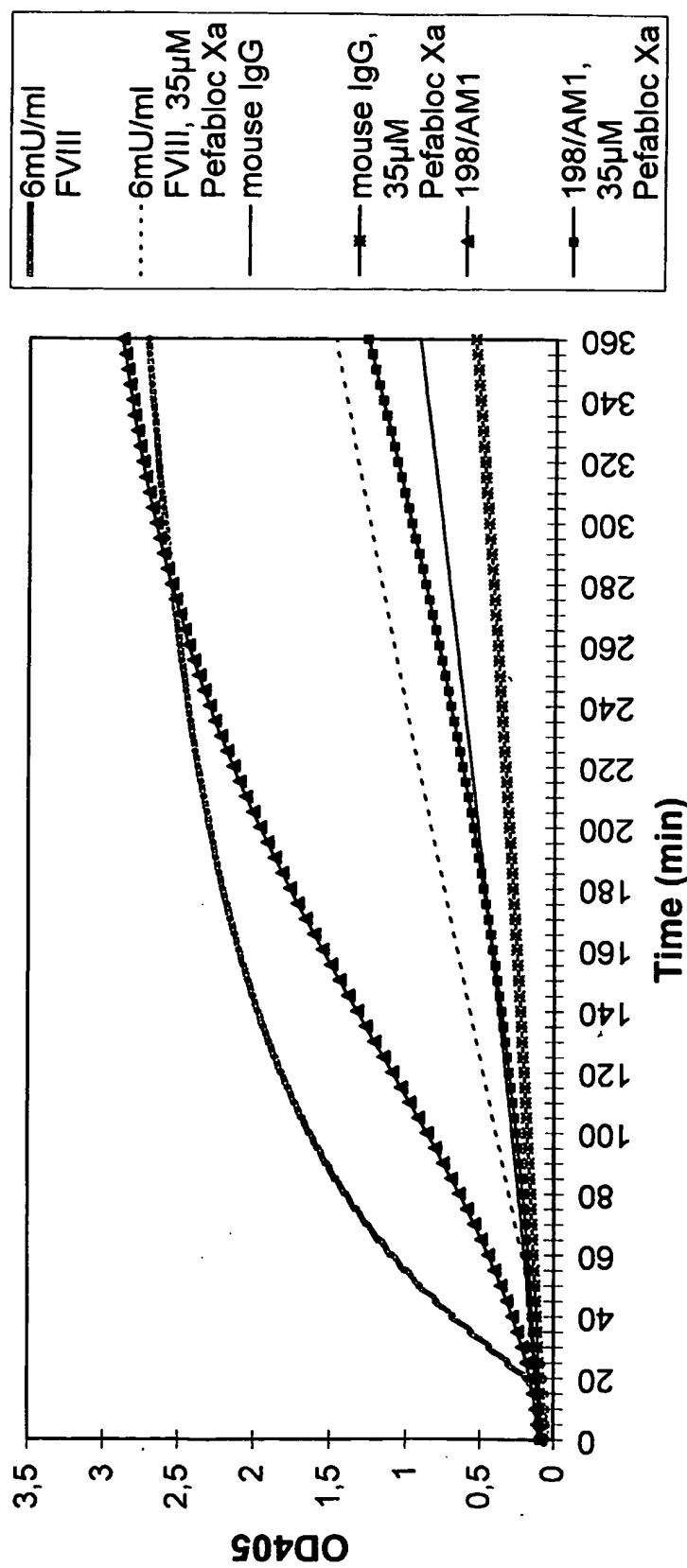


Fig. 7B

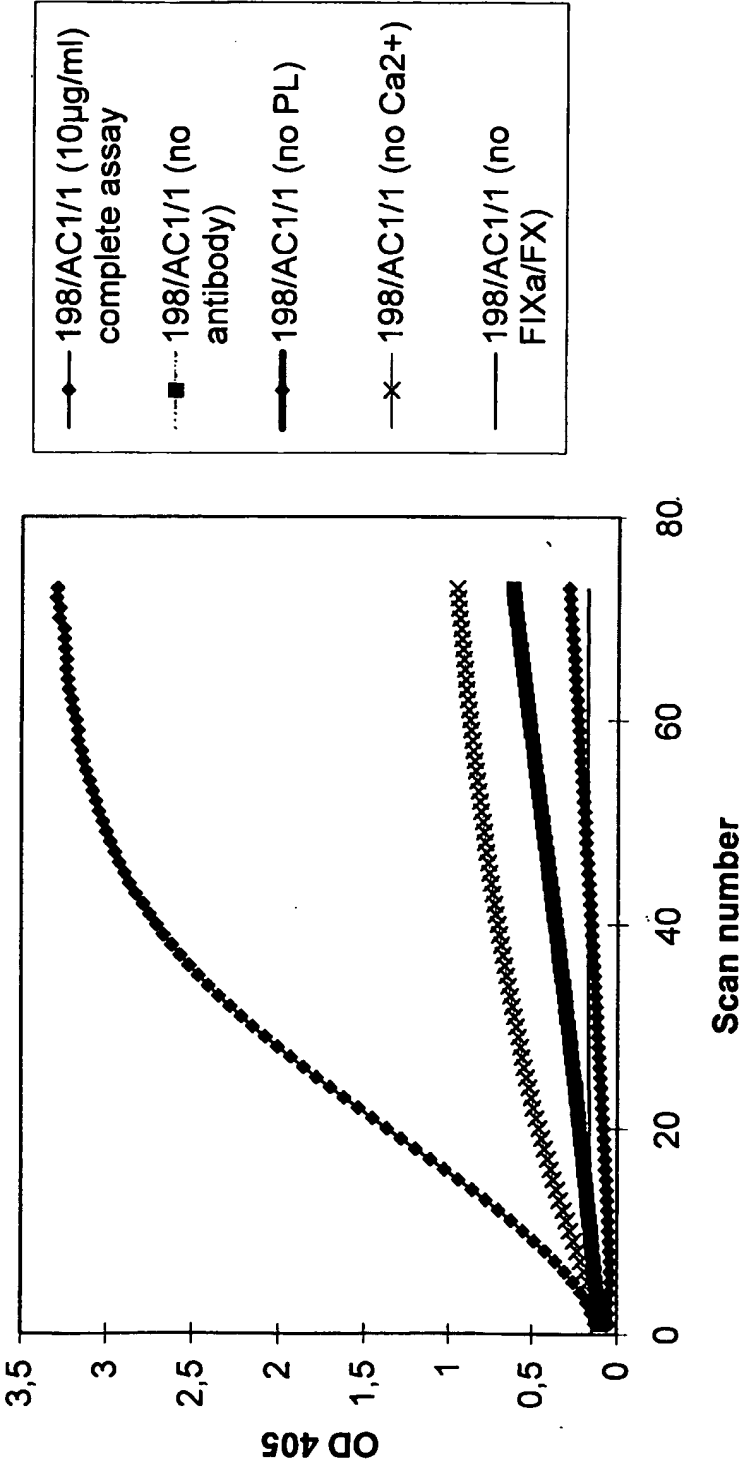


Fig. 8A

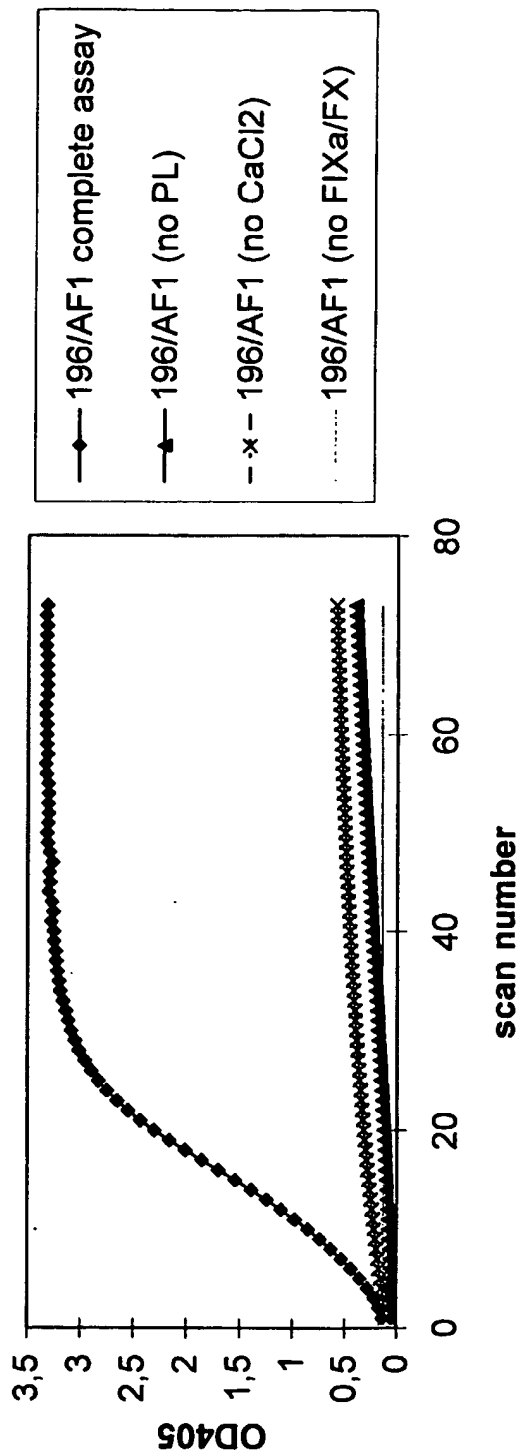


Fig. 8B



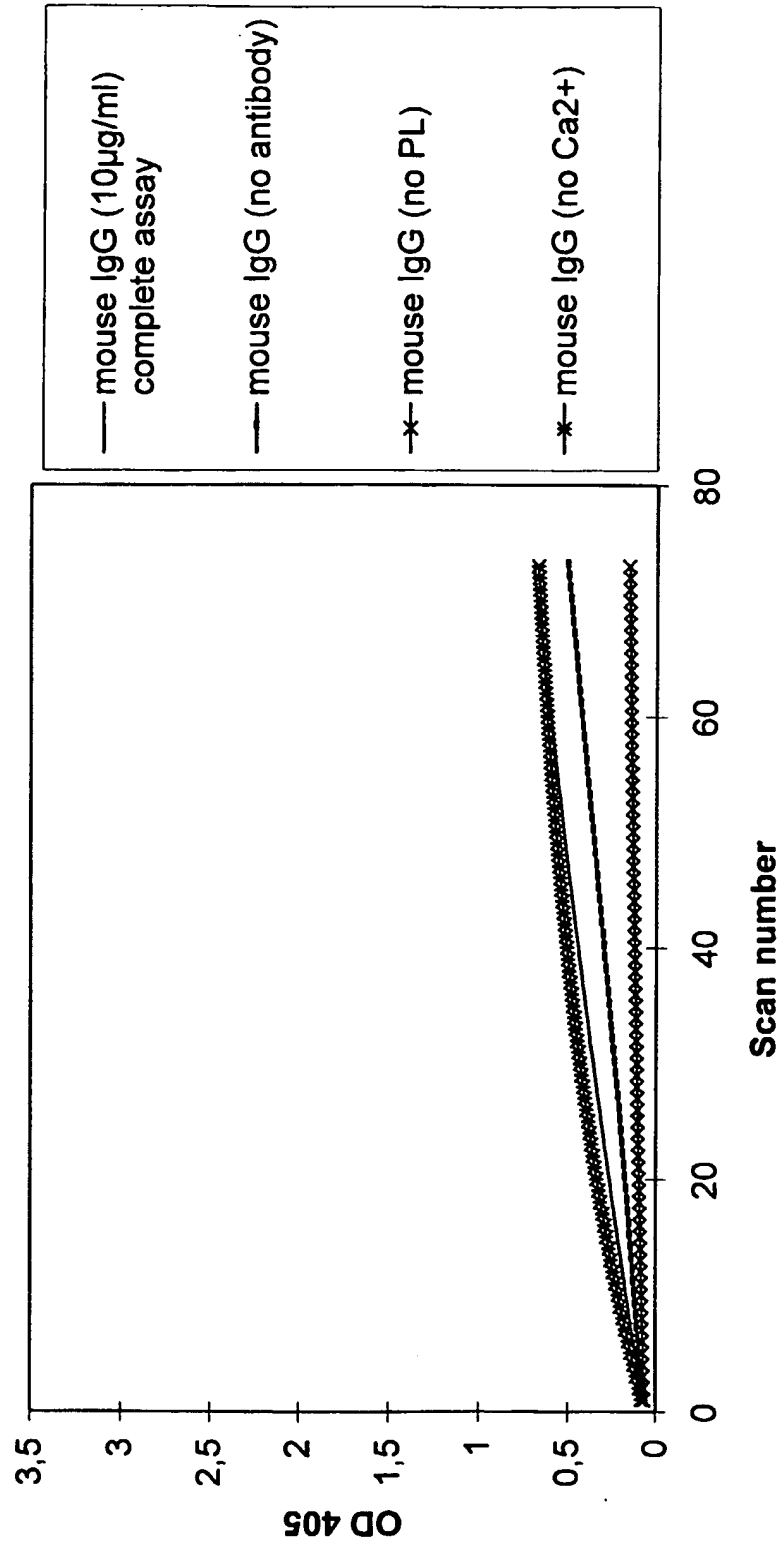


Fig. 8C

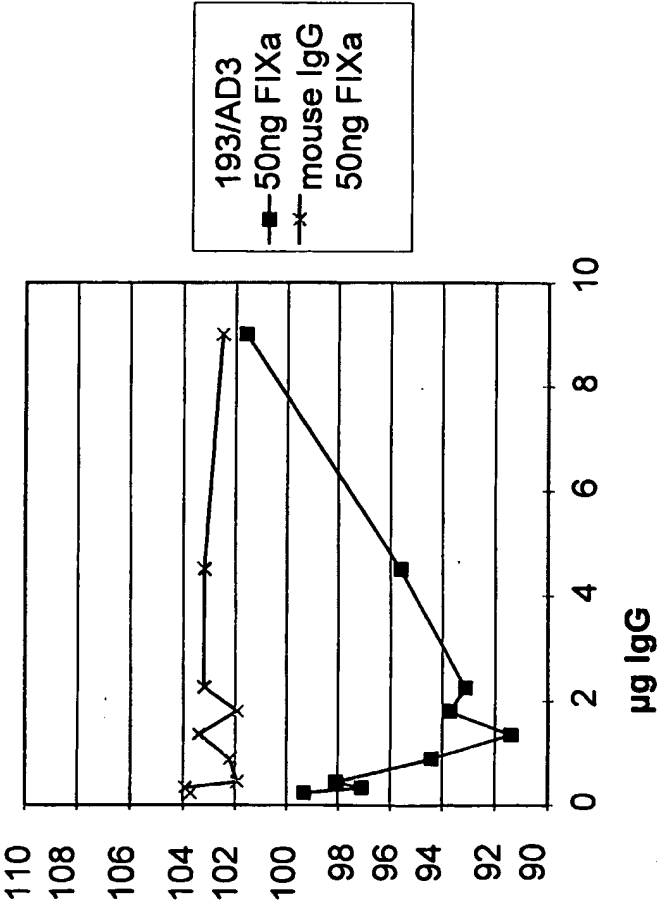


Fig.9

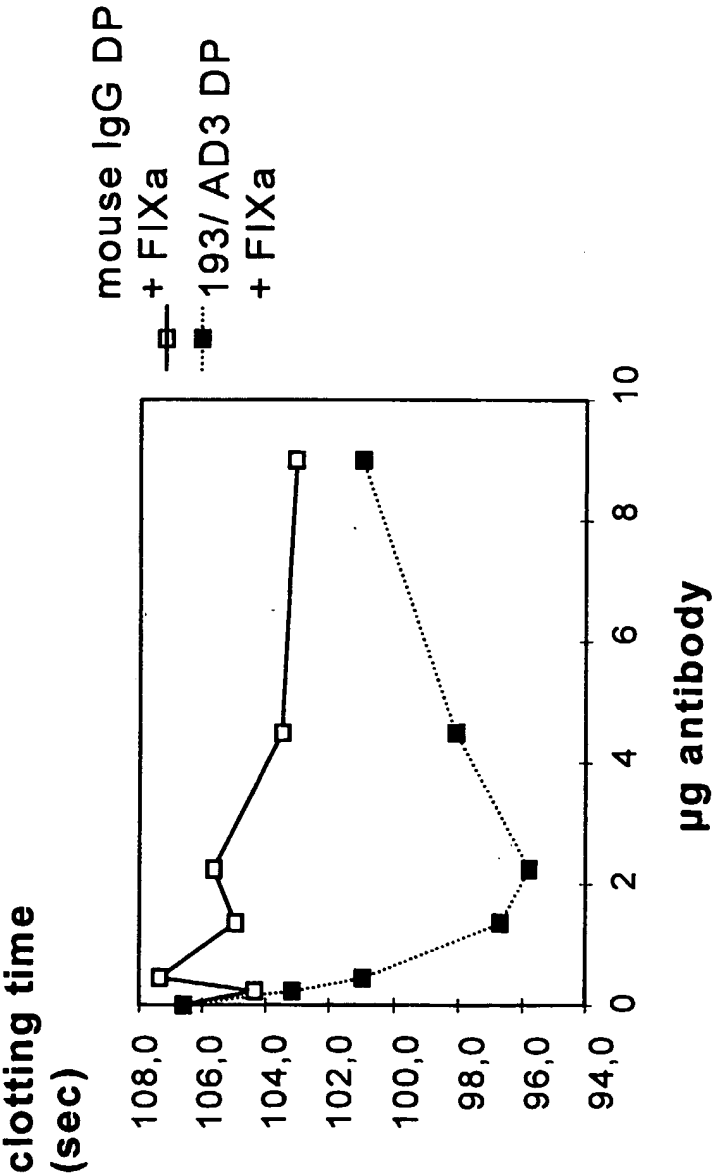


Fig. 10A

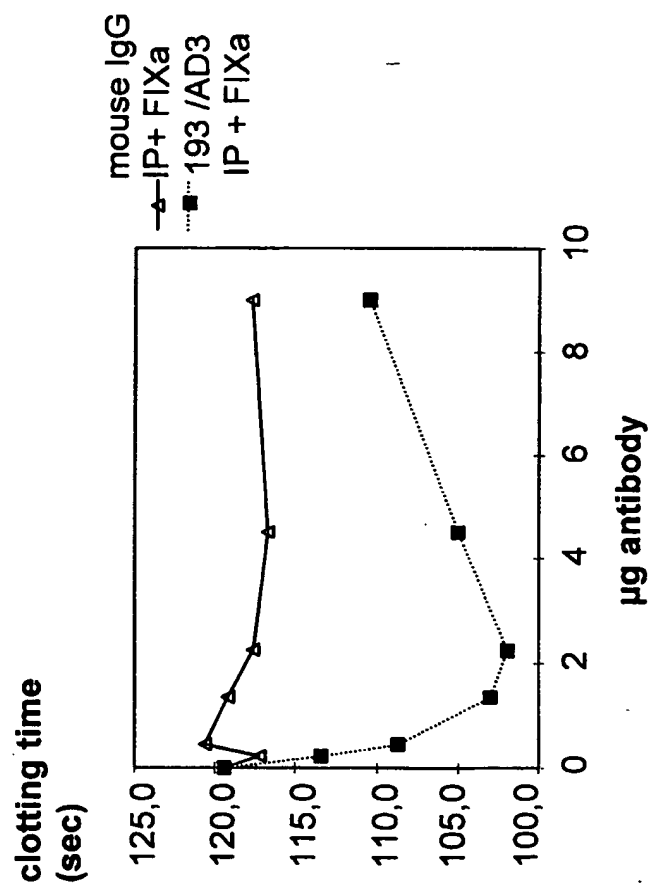


Fig. 10B

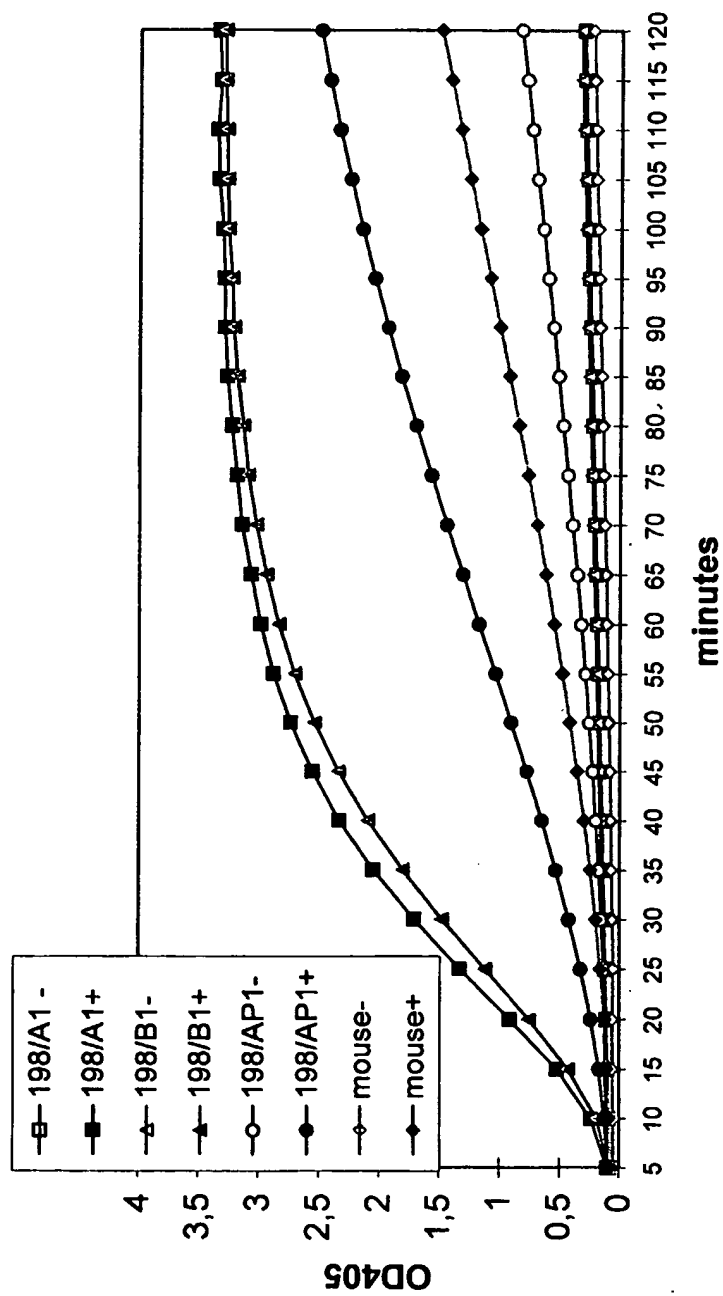


Fig.11

Mouse *V<sub>H</sub>* back primers (containing *Sfi*I-site):

VH1BACK-SfiI	5' C ATG CCA TGA CTC GCG GCC CAG CCG GCC ATG GCC SAG GTS MAR CTG CAG SAG TCW GG 3' (SEQ.ID.NO. 50)
VH1BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTT CAG GAG TCA GG 3' (SEQ.ID.NO. 51)
VH2BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAT GTG CAG CTT CAG GAG TCR GG 3' (SEQ.ID.NO. 52)
VH3BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTG CAG CTG AAG SAG TCA GG 3' (SEQ.ID.NO. 53)
VH4/6BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTY CAG CTG CAR CAR TCT GG 3' (SEQ.ID.NO. 54)
VH5/9BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG GTY CAR CTG CAG CAG YCT GG 3' (SEQ.ID.NO. 55)
VH7BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAR GTG AAG CTG GTG GAR TCT GG 3' (SEQ.ID.NO. 56)
VH8BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTT CAG CTT CAG CAG TCT GG 3' (SEQ.ID.NO. 57)
VH10BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC GAA GTG CAG CTG KTG GAG WCT GG 3' (SEQ.ID.NO. 58)
VH11BACKSfi	5' GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCC CAG ATC CAG TTG CTG CAG TCT GG 3' (SEQ.ID.NO. 59)

Fig. 12-1

Mouse *J<sub>H</sub>* forward primers (containing 1/2 linker-sequence and *AscI*-site):

VH1FOR2LiAsc	5' ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CGT GGT CCC TTG GCC CC 3' (SEQ.ID.NO. 60)
JH1FORLiAsc	5' ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC CGT GGT CCC 3' (SEQ.ID.NO. 61)
JH2FORLiAsc	5' ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC TGT GAG AGT GGT GCC 3' (SEQ.ID.NO. 62)
JH3FORLiAsc	5' ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGC AGA GAC AGT GAC CAG AGT CCC 3' (SEQ.ID.NO. 63)
JH4FORLiAsc	5' ACC GCC AGA GGC GCG CCC ACC TGA ACC GCC TCC ACC TGA GGA GAC GGT GAC TGA GGT TCC 3' (SEQ.ID.NO. 64)

---

IUPAC-Code: M=A/C, W=A/T, R=A/G, Y=C/T, S=C/G, K=G/T, H=A/C/T, D=A/G/T, V=A/C/G, B=T/C/G.

Fig. 12 -2

Primers for cloning mouse  $V_K$  genesMouse  $V_K$  back primers (containing *AscI*-site and  $\frac{1}{4}$  linker-sequence):

VK2BACK-LiAscI	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GAG CTC ACC CAG TCT CCA 3' (SEQ.ID.NO. 65)
VK1BACKLi Asc	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GTG ATG WCA CAG TCT CC 3' (SEQ.ID.NO. 66)
VK2BACKLi Asc	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAT GTT KTG ATG ACC CAA ACT CC 3' (SEQ.ID.NO. 67)
VK3BACKLi Asc	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAT ATT GTG ATR ACB CAG GCW GC 3' (SEQ.ID.NO. 68)
VK4BACKLi Asc	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GTG CTG ACM CAR TCT CC 3' (SEQ.ID.NO. 69)
VK5BACKLi Asc	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG SAA AWT GTK CTC ACC CAG TCT CC 3' (SEQ.ID.NO. 70)
VK6BACKLi Asc	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAY ATY VMG ATG ACM CAG WCT CC 3' (SEQ.ID.NO. 71)
VK7BACKLi Asc	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG CAA ATT GTT CTC ACC CAG TCT CC 3' (SEQ.ID.NO. 72)
VK8BACKLi Asc	5' GGT TCA GAT GGG CGC GCC TCT GGC GGT GGC GGA TCG TCA TTA TTG CAG GTG CTT GTG GG 3' (SEQ.ID.NO. 73)

Fig. 13-1



Mouse  $J_K$  forward primers (containing NotI-site):

JK1NOT10	5'	GAG	TCA	TTC	TGC	GGC	CGC	CCG	TTT	GAT	TTC	CAG	CTT	GGT	CCC	3'
		(SEQ.ID.NO. 74)														
JK2NOT10	5'	GAG	TCA	TTC	TGC	GGC	CGC	CCG	TTT	TAT	TTC	CAG	CTT	GGT	CCC	3'
		(SEQ.ID.NO. 75)														
JK3NOT10	5'	GAG	TCA	TTC	TGC	GGC	CGC	CCG	TTT	TAT	TTC	CAG	TCT	GGT	CCC	3'
		(SEQ.ID.NO.76)														
JK4NOT10	5'	GAG	TCA	TTC	TGC	GGC	CGC	CCG	TTT	TAT	TTC	CAA	CTT	TGT	CCC	3'
		(SEQ.ID.NO. 77)														
JK5NOT10	5'	GAG	TCA	TTC	TGC	GGC	CGC	CCG	TTT	CAG	CTC	CAG	CTT	GGT	CCC	3'
		(SEQ.ID.NO. 78)														

---

IUPAC-Code: K=G/T, M=A/C, W=A/T, R=A/G, Y=C/T, S=C/G, H=A/C/T, D=A/G/T, V=A/C/G, B=T/C/G.

Fig. 13-2

VH

+1 E V K L V E S G P E L K K P G  
 1 GAG GTG AAG CTG GTG GAG TCT GGA CCT GAG CTG AAG AAG CCT GGA  
 +1 E T V K I S C K A S G Y I F T  
 46 GAG ACA GTC AAG ATC TCC TGC AAG GCT TCT GGG TAT ATC TTC ACA  
 +1 N Y G M N W V K Q A P G K G L  
 91 AAC TAT GGA ATG AAC TGG GTG AAG CAG GCT CCA GGA AAG GGT TTA  
 +1 K W M G W I N T Y T G E P T Y  
 136 AAG TGG ATG GGC TGG ATA AAC ACC TAC ACT GGA GAG CCA ACA TAT  
 +1 A D D F K G R F A F S L E T S  
 181 GCT GAT GAC TTC AAG GGA CGG TTT GCC TTC TCT TTG GAA ACC TCT  
 +1 A S T A Y L Q I N N L K N E D  
 226 GCC AGC ACT GCC TAT TTG CAG ATC AAC AAC CTC AAA AAT GAG GAC  
 +1 T A T Y F C A L Y G N S P K G  
 271 ACG GCT ACA TAT TTC TGT GCA TTA TAT GGT AAC TCC CCT AAG GGG

*linker*

+1 F A Y W G Q G T L V T V S A G  
 316 TTT GCT TAC TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA GGT

VL

+1 G G G S G G R A S G G G G S D  
 361 GGA GGC GGT TCA GGT GGG CGC GCC TCT GGC GGT GGC GGA TCG GAT  
 +1 I Q M T Q S P K F L L V S A G  
 406 ATT CAG ATG ACA CAG TCT CCC AAA TTC CTG CTT GTA TCA GCA GGA

Fig. 14-1

+1 D R V T I T C K A S Q S V S N  
451 GAC AGG GTT ACC ATA ACC TGC AAG GCC AGT CAG AGT GTG AGT AAT  
  
+1 D V A W Y Q Q K P G Q S P K L  
496 GAT GTA GCT TGG TAC CAA CAG AAG CCG GGG CAG TCT CCT AAA CTA  
  
+1 L M Y Y A S N R Y T G V P D R  
541 CTG ATG TAC TAT GCA TCC AAT CGC TAC ACT GGA GTC CCT GAT CGC  
  
+1 F T G S G Y G T D F T F T I S  
586 TTC ACT GGC AGT GGA TAT GGG ACG GAT TTC ACT TTC ACC ATC AGC  
  
+1 T V Q A E D L A V Y F C Q Q D  
631 ACT GTG CAG GCT GAA GAC CTG GCA GTT TAT TTC TGT CAG CAG GAT  
  
+1 Y G S P P T F G G G T K L E I  
676 TAT GGC TCT CCT CCC ACG TTC GGA GGG GGC ACC AAG CTG GAA ATT  
  
+1 K R  
721 AAA CGG

Fig. 14-2

```

VH
+1 E V Q L V E S G G G L V K P G
1 GAA GTG CAG CTG GTG GAG TCT GGG GGA GGC CTA GTG AAG CCT GGA

+1 G S L K L S C A A S G F T F S
46 GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT

+1 T Y T M S W V R Q T P E K R L
91 ACC TAT ACC ATG TCT TGG GTT CGC CAG ACT CCG GAG AAG AGG CTG

+1 E W V A T I S S G G S Y T Y Y
136 GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT AGT TAC ACC TAC TAT

+1 P D S V R G R F T I S R D N A
181 CCA GAC AGT GTG AGG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC

+1 K N T L Y L Q M S S L K S E D
226 AAG AAC ACC CTG TAC CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC

+1 T A M Y Y C T R D G G H G Y G
271 ACA GCC ATG TAT TAC TGT ACA AGA GAT GGG GGA CAC GGG TAC GGT

+1 S S F D Y W G Q G T T L' T V S
316 AGT AGC TTT GAC TAC TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC

linker
+1 S G G G S G G R A S G G G
361 TCA GGT GGA GGC GGT TCA GGT GGG CGC GCC TCT GGC GGT GGC GGA

VL
+1 S Q I V L T Q S P L S L P V S
406 TCG CAA ATT GTG CTC ACC CAG TCT CCA CTC TCC CTG CCT GTC AGT

```

Fig. 15-1

```

+1   L   G   D   Q   A   S   I   S   C   R   S   S   Q   S   I
451  CTT GGA GAT CAA GCC TCC ATC TCT TGC AGA TCT AGT CAG AGC ATT

+1   V   H   S   N   G   N   T   Y   L   E   W   Y   L   Q   K
496  GTA CAT AGT AAT GGA AAC ACC TAT TTA GAA TGG TAC CTG CAG AAA

+1   P   G   Q   S   P   K   L   L   I   Y   K   V   S   N   R
541  CCA GGC CAG TCT CCA AAG CTC CTG ATC TAC AAA GTT TCC AAC CGA

+1   F   S   G   V   P   D   K   F   S   G   S   G   S   G   T
586  TTT TCT GGG GTC CCA GAC AAA TTC AGT GGC AGT GGA TCA GGG ACA

+1   D   F   T   L   K   I   S   R   V   E   A   E   D   L   G
631  GAT TTC ACA CTC AAG ATC AGC AGA GTG GAG GCT GAG GAT CTG GGA

+1   V   Y   Y   C   F   Q   G   S   H   V   P   W   T   F   G
676  GTT TAT TAC TGC TTT CAA GGT TCA CAT GTT CCG TGG ACG TTC GGT

1    G   G   T   K   L   E   I   K   R
721  GGA GGC ACC AAG CTG GAA ATC AAA CGG

```

Fig. 15-2

```

+1 E V Q L Q E S G G L V K P G
1 GAG GTG CAG CTT CAG GAG TCA GGG GGA GGC TTA GTG AAG CCT GGA

+1 G S L K L S C A A S G F T F S
46 GGG TCC CTG AAA CTC TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT

+1 S Y T M S W V R Q T P E K R L
91 AGC TAT ACC ATG TCT TGG GTT CGC CAG ACT CCG GAG AAG AGG CTG

+1 E W V A T I S S G S S T Y Y
136 GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT AGT TCC ACC TAC TAT

+1 P D S V K G R F T I S R D N A
181 CCA GAC AGT GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC

+1 K N T L Y L Q M S S L R S E D
226 AAG AAC ACC CTG TAC CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC

+1 T A M Y Y C T R E G G G F T V
271 ACA GCC ATG TAT TAC TGT ACA AGA GAG GGG GGT GGT TTC ACC GTC

+1 N W Y F D V W G A G T L V T V
316 AAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACT CTG GTC ACT GTC

linker
+1 S A G G G G S G G R A S G G G
361 TCT GCA GGT GGA GGC GGT TCA GGT GGG CGC GCC TCT GGC GGT GGC

VL
+1 G S E N V L T Q S P A S L A V
406 GGA TCG GAA AAT GTG CTC ACC CAG TCT CCA GCT TCT TTG GCT GTG

```

Fig. 16-1

```

+1 S L G Q R A T I S C R A S E S
451 TCT CTA GGG CAG AGG GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT

+1 V D S Y G Y N F M H W Y Q Q I
496 GTT GAT AGT TAT GGC TAT AAT TTT ATG CAC TGG TAT CAG CAG ATA

+1 P G Q P P K L L I Y R A S N L
541 CCA GGA CAG CCA CCC AAA CTC CTC ATC TAT CGT GCA TCC AAC CTA

+1 E S G I P A R F S G S G S R T
586 GAG TCT GGG ATC CCT GCC AGG TTC AGT GGC AGT GGG TCT AGG ACA

+1 D F T L T I N P V E A D D V A
631 GAC TTC ACC CTC ACC ATT AAT CCT GTG GAG GCT GAT GAT GTT GCA

+1 T Y Y C Q Q S N E D P L T F G
676 ACC TAT TAC TGT CAG CAA AGT AAT GAG GAT CCG CTC ACG TTC GGT

+1 T G T R L E I K R
721 ACT GGG ACC AGA CTG GAA ATA AAA CGG

```

Fig.16 -2

VH

+1 E V Q L Q E S G G G G L V K P G G S L K L  
 1 GAG GTG CAG CTT CAG GAG TCA GGG GGA CCT GGA GGG TCC CTG AAA CTC  
 CTC CAC GTC GAA GTC CTC AGT CCC CCT CCG AAT CAC TTC GGA CCG CCC AGG GAC TTT GAG

+1 S C A A S G G F I F S S Y T M S W V R Q T  
 61 TCC TGT GCA GCC TCT GGA TTC ATT TTT AGT AGT TAT ACC ATG TCT TGG GTT CGC CAG ACT  
 AGG ACA CGT CGG AGA CCT AAG TAA AAA TCA TCA ATA TGG TAC AGA ACC CAA GCG GTC TGA

+1 P E K R L E W V A T I S S G G S S T Y Y  
 121 CCG GAG AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT GGT GGT TCC ACC TAC TAT  
 GGC CTC TTC TCC GAC CTC ACC CAG CGT TGG TAA TCA TCA CCA CCA TCA AGG TGG ATG ATA

+1 P D S V K G R F T I S R D N A K N T L Y  
 181 CCA GAC AGT GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC  
 GGT CTG TCA CAC TTC CCG GCT AAG TGG TAG AGG TCT CTG TTA CCG TTC TTG TGG GAC ATG

+1 L Q M S S L K S E D T A M Y H C T R E G  
 241 CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC ACA GCC ATG TAT CAC TGT ACA AGA GAG GGG  
 GAC GTT TAC TCG TCA GAC TTC AGA CTC CTG TGT CGG TAC ATA GTG ACA TGT TCT CTC CCC

+1 G G Y Y V N W Y F D V W G A G T T L T V  
 301 GGT GGT TAT TAC GTC AAC TGG TAC TTC GAT GTC TGG GGC GCA GGC ACC ACT CTC ACA GTC  
 CCA CCA ATA ATG CAG TTG ACC ATG AAG CTA CAG ACC CCG CGT CCG TGG TGA GAG TGT CAG

linker

VL

+1 S S G G G G S S G G R A S G G G G S D I E  
 361 TCC TCA GGT GGA GGC GGT TCA GGT GGC CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GAG  
 AGG AGT CCA CCT CCG CCA AGT CCA CCC GCG CGG AGA CCG CCA CCG CCT AGC CTG TAA CTC

+1 L T Q S P A S L A V S L G Q R A T I S C  
 421 CTC ACN CAG TCT CCA GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATA TCC TGC  
 GAG TGN GTC AGA GGT CGA AGA AAC CGA CAC AGA GAT CCC GTC TCC CGG TGG TAT AGG ACC

Fig. 17-1



```

+1 R A S E S V D S Y G K S F M H W Y Q Q K
481 AGA GCC AGT GAA AGT GTT GAT AGT TAT GGC AAG AGT TTT ATG CAC TGG TAC CAG CAG AAA
TCT CGG TCA CTT TCA CAA CTA TCA ATA CCG TTC TCA AAA TAC GTG ACC ATG GTC GTC TTT
+1 P G Q P P K L L I Y R A S N L E S G I P
541 CCA GGG CAG CCA CCC AAA CTC CTC ATC TAT CGT GCA TCC AAC CTA GAA TCT GGG ATC CCT
GGT CCC GTC GGT GGG TTT GAG GAG TAG ATA GCA CGT AGG TTG GAT CTT AGA CCC TAG GGA
+1 A R F S S G S G S R T D F T L T I N P V E
601 GCC AGG TTC AGT GGC AGT GGC TCT AGG ACA GAC TTC ACC CTC ACC ATT AAT CCT GTG GAG
CGG TCC AAG TCA CCG TCA CCC AGA TCC TGT CTG AAG TGG GAG TGG TAA TTA GGA CAC CTC
+1 A D D V A T Y Y C Q Q S N E D P L T F G
661 GCT GAT GAT GTT GCN ACC TAT TAC TGT CAG CAA AGT AAT GAG GAT CCC CTC ACG TTC GGT
CGA CTA CTA CAA CGN TGG ATA ATG ACA GTC GTT TCA TTA CTC CTA GGG GAG TGC AAG CCA
+1 A G T R L E I K R
721 GCT GGG ACC AGA CTG GAA ATA AAA CGG
CGA CCC TGG TCT GAC CTT TAT TTT GCC

```

Fig. 17-2

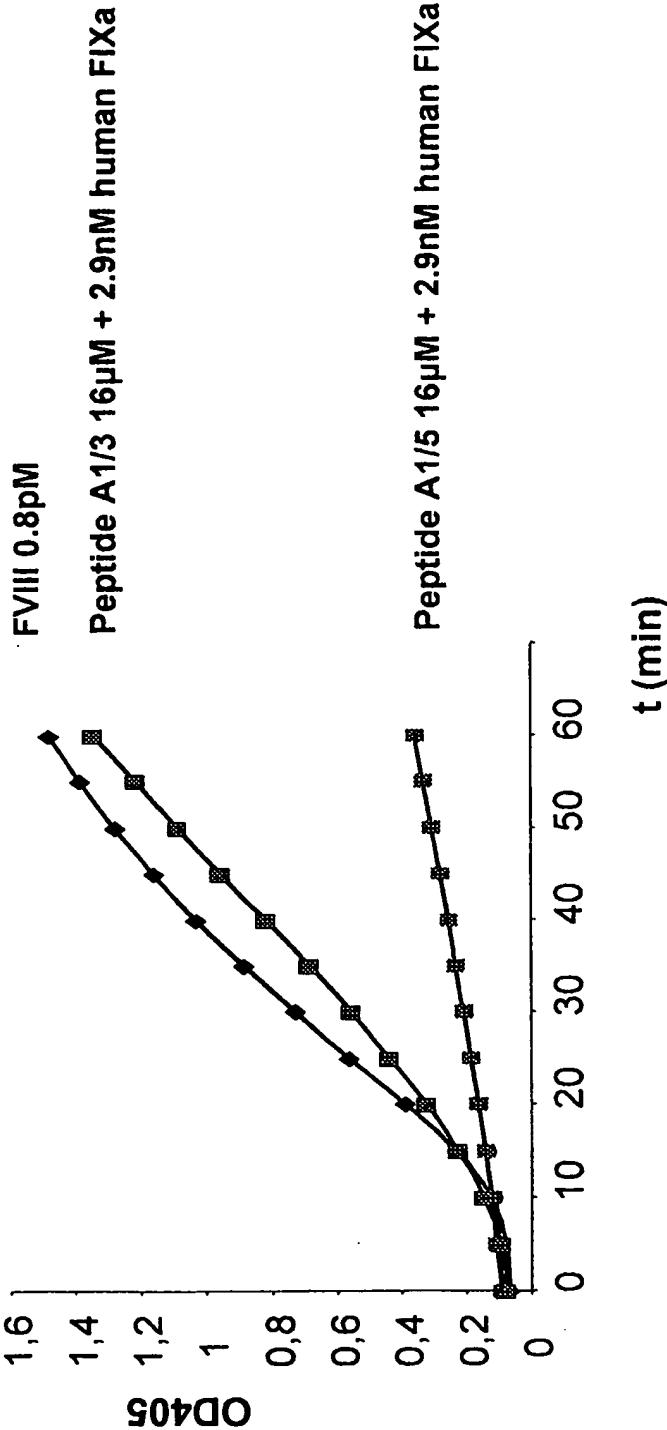


Fig. 18

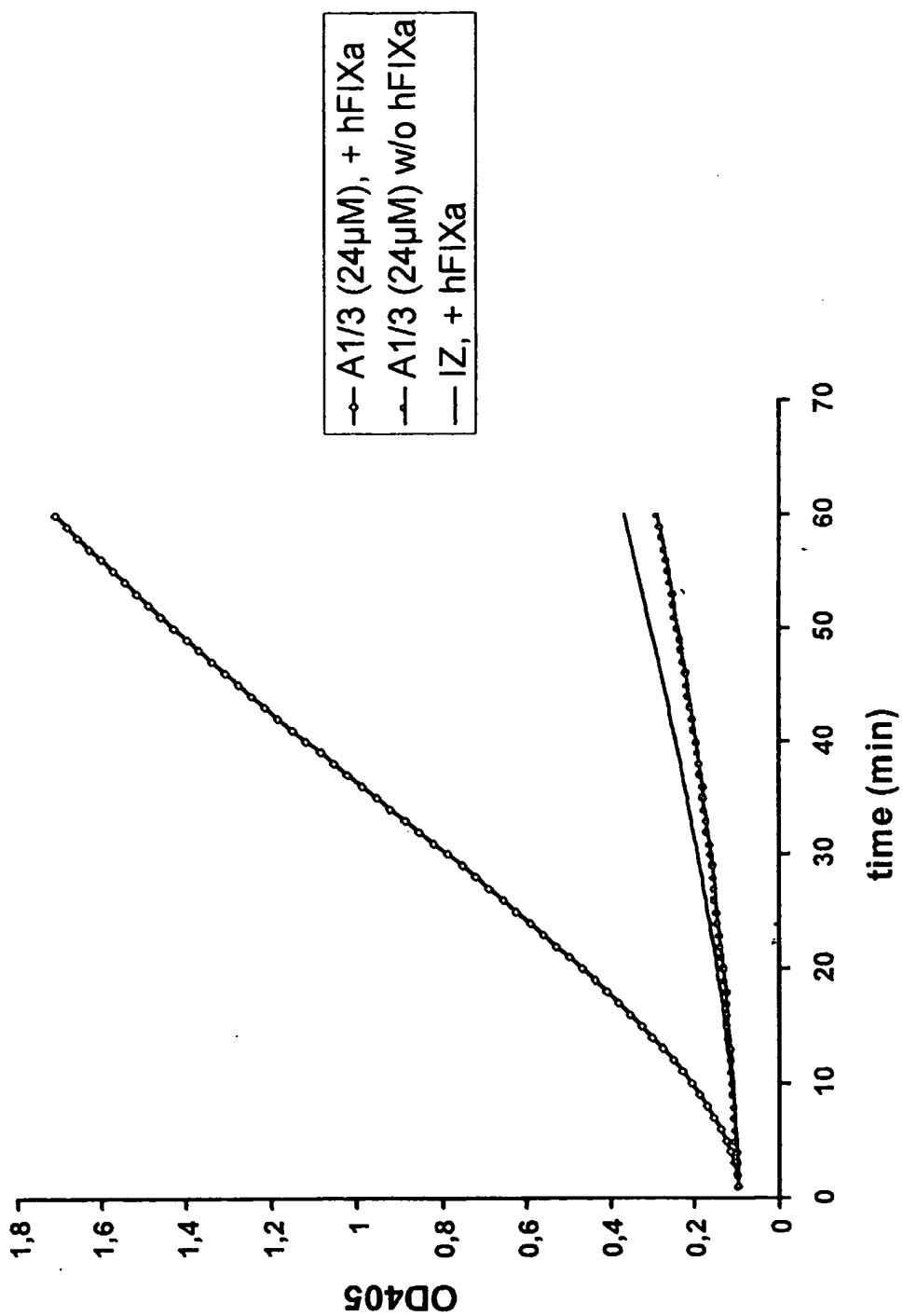


Fig. 19

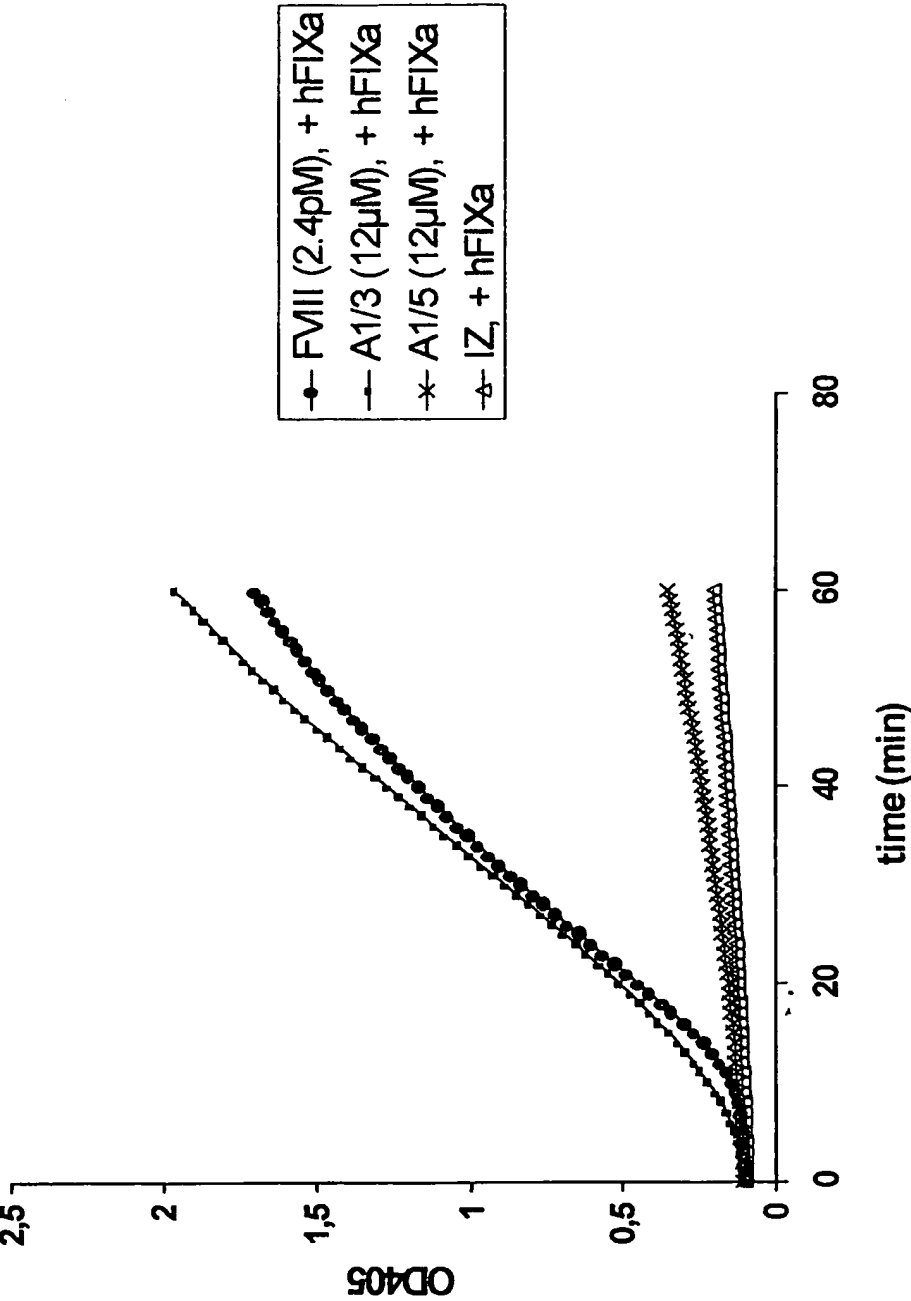


Fig. 20

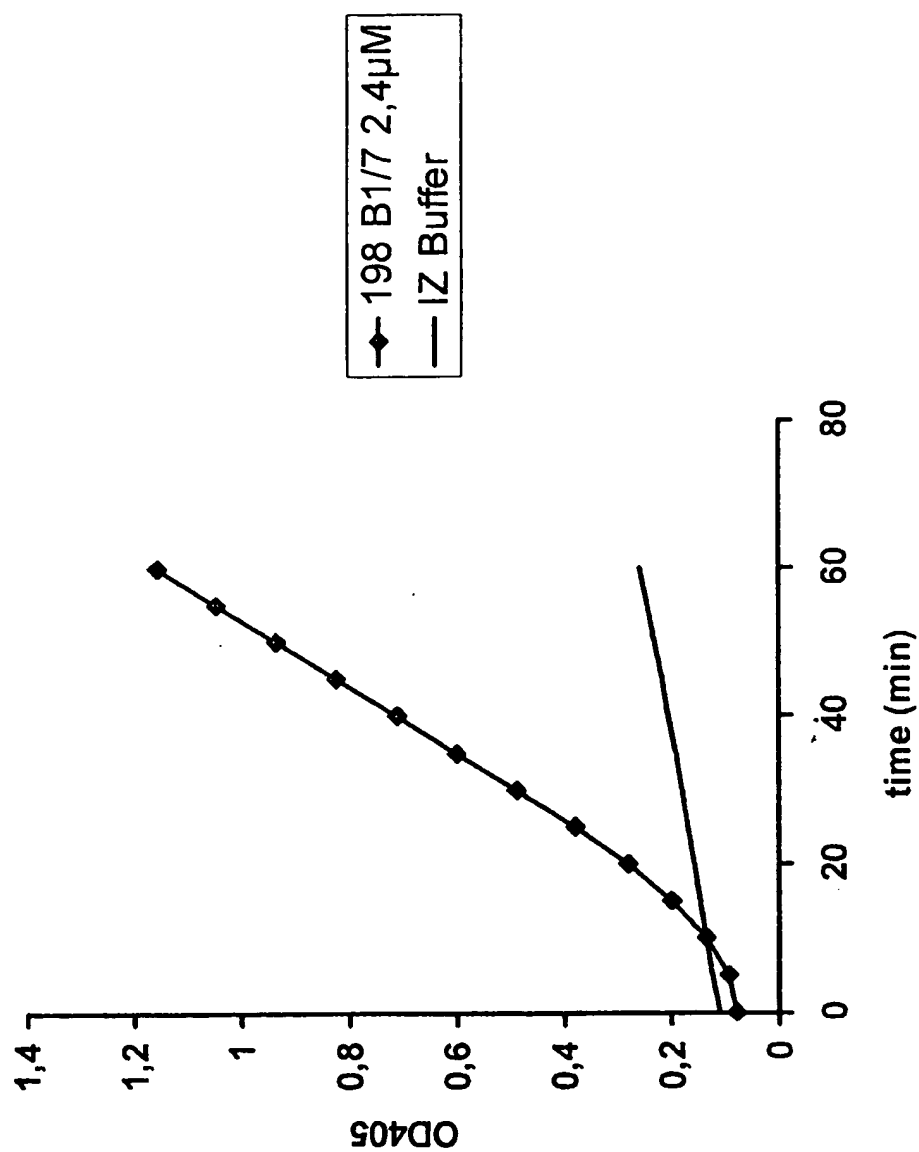


Fig. 21

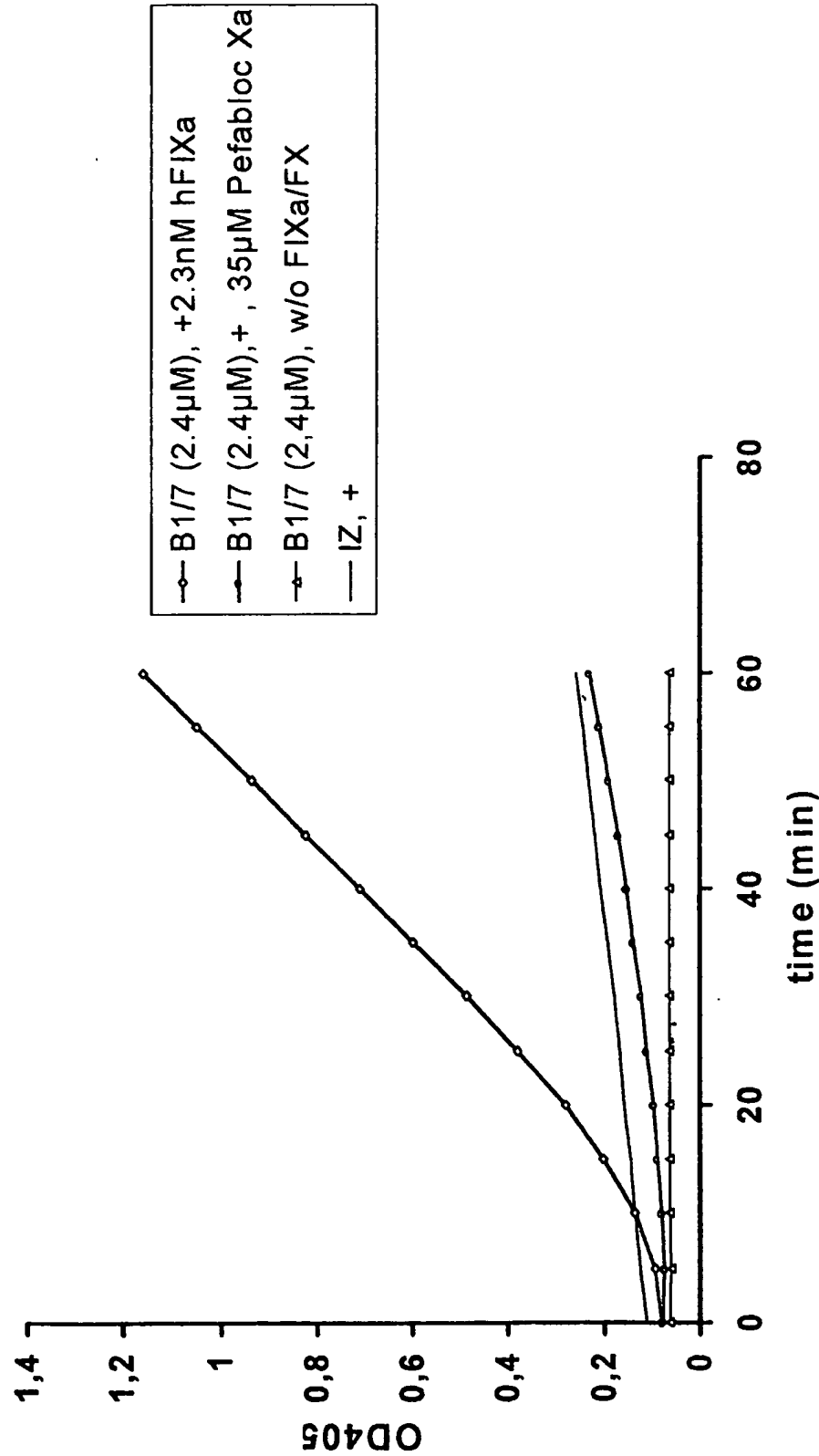


Fig. 22

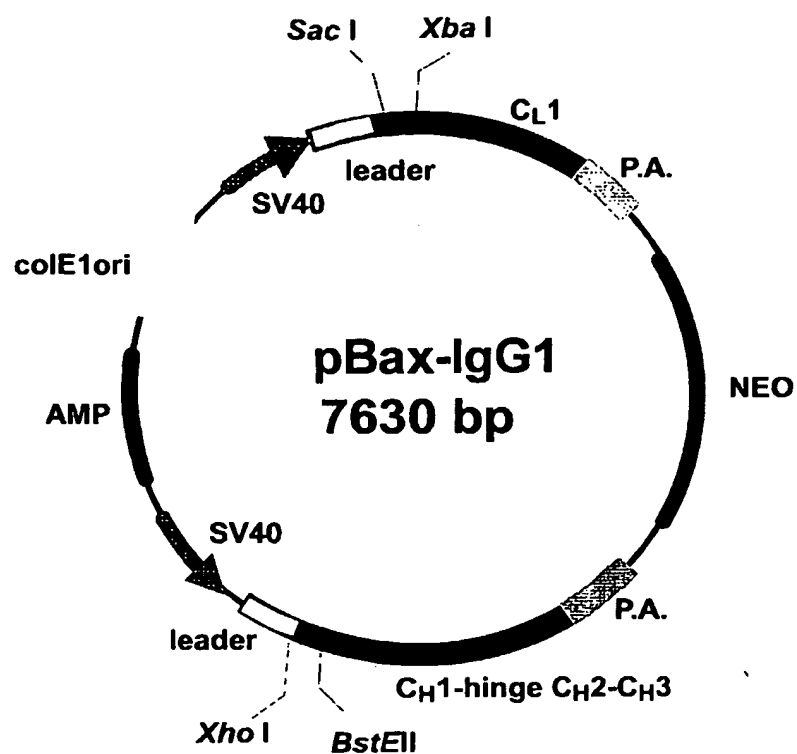


Figure 23

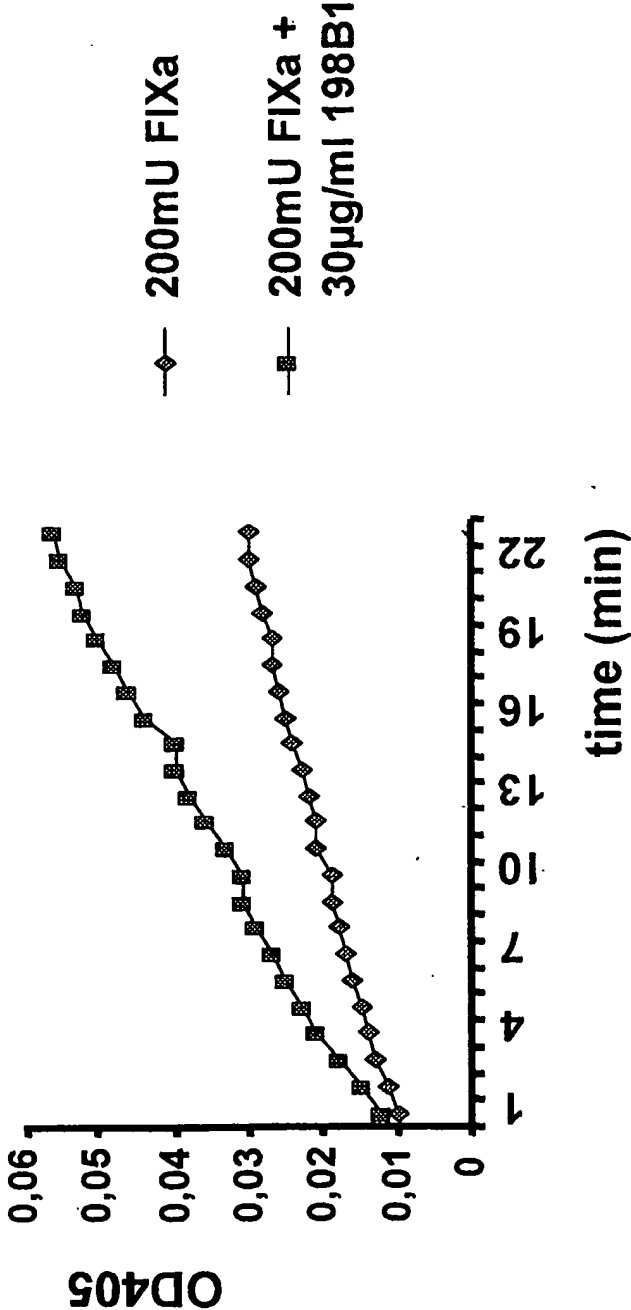


Fig. 24A



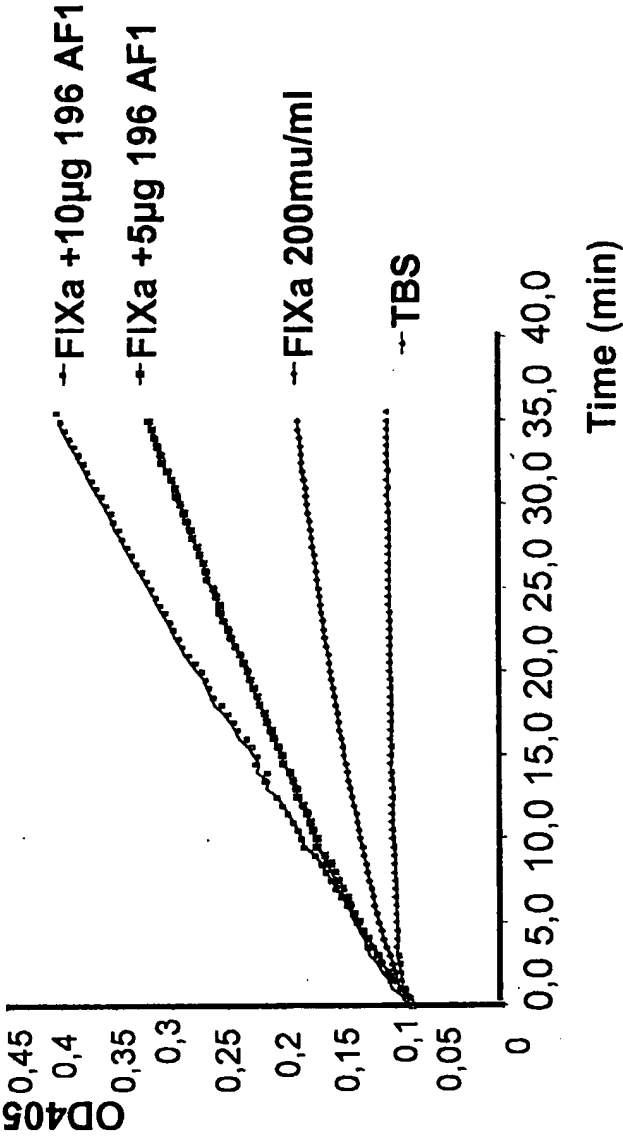


Fig. 24B

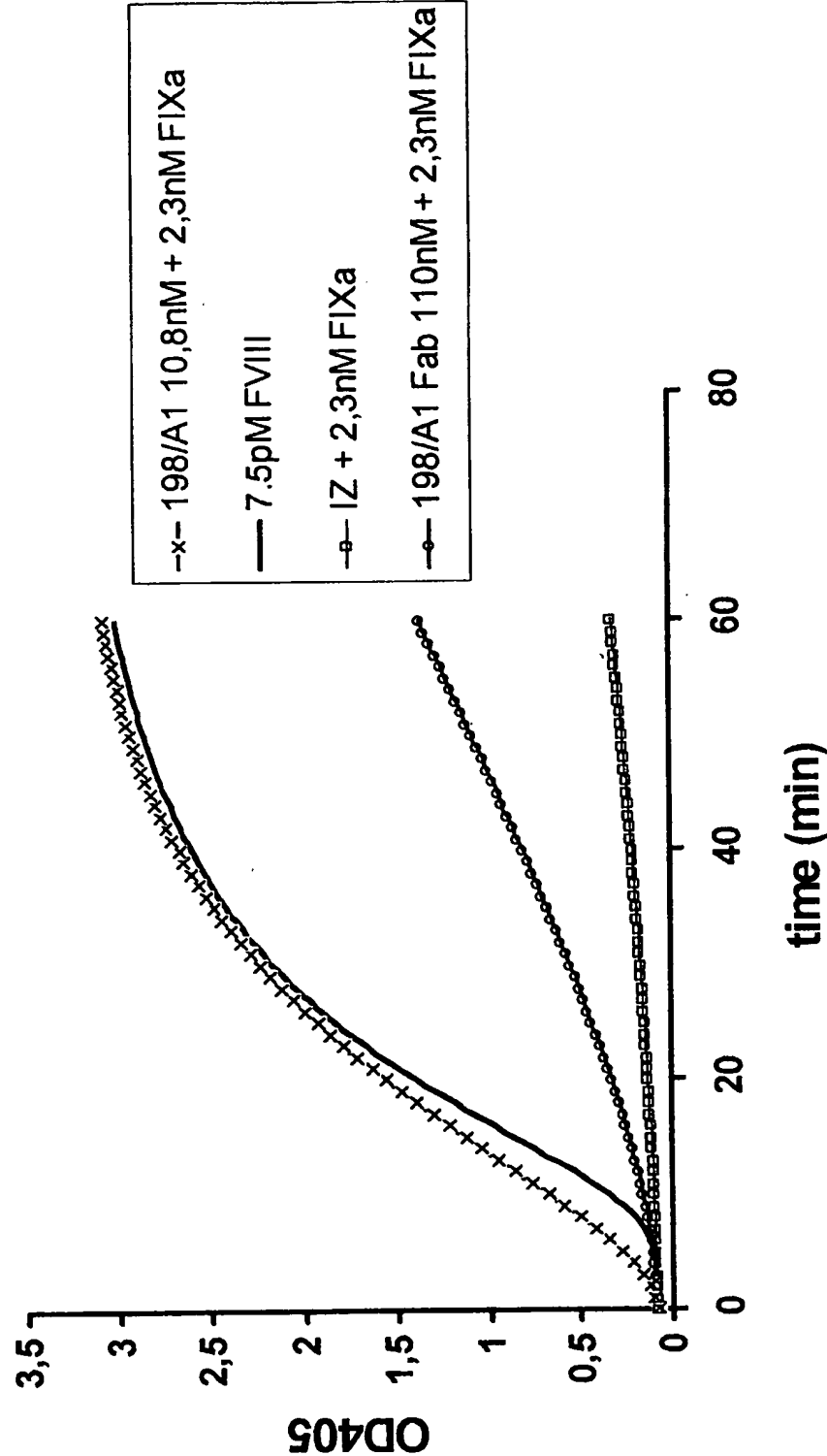


Fig. 25

PelB-leader

```

+1  M   K   Y   L   L   P   T   A   A   A   G   L   L   L
1   ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA
    TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT

```

VH

```

+1  L   A   A   Q   P   A   M   A   E   V   K   L   V   E
43  CTC GCG GCC CAG CCG GCC ATG GCG GAG GTG AAG CTG GTG GAG
    GAG CGC CGG GTC GGC CGG TAC CGC CTC CAC TTC GAC CAC CTC

```

```

+1  S   G   G   G   L   V   K   P   G   G   S   L   K   L
85  TCT GGG GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC
    AGA CCC CCT CCG AAT CAC TTC GGA CCT CCC AGG GAC TTT GAG

```

```

+1  S   C   A   A   S   G   F   T   F   S   S   Y   T   M
127 TCC TGT GCA GCC TCT GGA TTC ACT TTC AGT AGC TAT ACC ATG
    AGG ACA CGT CGG AGA CCT AAG TGA AAG TCA TCG ATA TGG TAC

```

```

+1  S   W   V   R   Q   T   P   E   K   R   L   E   W   V
169 TCT TGG GTT CGC CAG ACT CCG GAG AAG AGG CTG GAG TGG GTC
    AGA ACC CAA GCG GTC TGA GGC CTC TTC TCC GAC CTC ACC CAG

```

```

+1  A   T   I   S   S   G   G   S   S   T   Y   Y   P   D
211 GCA ACC ATT AGT AGT GGN GGT AGT TCC ACC TAC TAT CCA GAC
    CGT TGG TAA TCA TCA CCN CCA TCA AGG TGG ATG ATA GGT CTG

```

```

+1  S   V   K   G   R   F   T   I   S   R   D   N   A   K
253 AGT GTG AAG GGC CGA TTC ACC ATC TCC AGA GAC AAT GCC AAG
    TCA CAC TTC CCG GCT AAG TGG TAG AGG TCT CTG TTA CGG TTC

```

```

+1  N   T   L   Y   L   Q   M   S   S   L   R   S   E   D
295 AAC ACC CTG TAC CTG CAA ATG AGC AGT CTG AGG TCT GAG GAC
    TTG TGG GAC ATG GAC GTT TAC TCG TCA GAC TCC AGA CTC CTG

```

```

+1  T   A   M   Y   Y   C   T   R   E   G   G   G   F   T
337 ACA GCC ATG TAT TAC TGT ACA AGA GAG GGG GGT GGT TTC ACC
    TGT CGG TAC ATA ATG ACA TGT TCT CTC CCC CCA CCA AAG TGG

```

```

+1  V   N   W   Y   F   D   V   W   G   A   G   T   S   V
379 GTC AAC TGG TAC TTC GAT GTC TGG GGC GCA GGA ACC TCA GTC
    CAG TTG ACC ATG AAG CTA CAG ACC CCG CGT CCT TGG AGT CAG

```

Linker

```

+1  T   V   S   S   G   G   G   G   S   G   G   R   A   S
421 ACC GTC TCC TCA GGT GGA GGC GGT TCA GGT GGG CGC GCC TCT
    TGG CAG AGG AGT CCA CCT CCG CCA AGT CCA CCC GCG CGG AGA

```

Fig. 26-1

							VL								
+1	G	G	G	G	S	D	I	V	L	T	Q	S	P	A	
463	GGC	GGT	GGC	GGA	TCG	GAC	ATT	GTG	CTG	ACA	CAG	TCT	CCA	GCT	
	CCG	CCA	CCG	CCT	AGC	CTG	TAA	CAC	GAC	TGT	GTC	AGA	GGT	CGA	
+1	S	L	A	V	S	L	G	Q	R	A	T	I	S	C	
505	TCT	TTG	GCT	GTG	TCT	CTA	GGG	CAG	AGG	GCC	ACC	ATA	TCC	TGC	
	AGA	AAC	CGA	CAC	AGA	GAT	CCC	GTC	TCC	CGG	TGG	TAT	AGG	ACG	
+1	R	A	S	E	S	V	D	S	Y	G	Y	N	F	M	
547	AGA	GCC	AGT	GAA	AGT	GTT	GAT	AGT	TAT	GGC	TAT	AAT	TTT	ATG	
	TCT	CGG	TCA	CTT	TCA	CAA	CTA	TCA	ATA	CCG	ATA	TTA	AAA	TAC	
+1	H	W	Y	Q	Q	I	P	G	Q	P	P	K	L	L	
589	CAC	TGG	TAT	CAG	CAG	ATA	CCA	GGA	CAG	CCA	CCC	AAA	CTC	CTC	
	GTG	ACC	ATA	GTC	GTC	TAT	GGT	CCT	GTC	GGT	GGG	TTT	GAG	GAG	
+1	I	Y	R	A	S	N	L	E	S	G	I	P	A	R	
631	ATC	TAT	CGT	GCA	TCC	AAC	CTA	GAG	TCT	GGG	ATC	CCT	GCC	AGG	
	TAG	ATA	GCA	CGT	AGG	TTG	GAT	CTC	AGA	CCC	TAG	GGA	CGG	TCC	
+1	F	S	G	S	G	S	R	T	D	F	T	L	T	I	
673	TTC	AGT	GGC	AGT	GGG	TCT	AGG	ACA	GAC	TTC	ACC	CTC	ACC	ATT	
	AAG	TCA	CCG	TCA	CCC	AGA	TCC	TGT	CTG	AAG	TGG	GAG	TGG	TAA	
+1	N	P	V	E	A	D	D	V	A	T	Y	Y	C	Q	
715	AAT	CCT	GTG	GAG	GCT	GAT	GAT	GTT	GCA	ACC	TAT	TAC	TGT	CAG	
	TTA	GGA	CAC	CTC	CGA	CTA	CTA	CAA	CGT	TGG	ATA	ATG	ACA	GTC	
+1	Q	S	N	E	D	P	L	T	F	G	T	G	T	R	
757	CAA	AGT	AAT	GAG	GAT	CCG	CTC	ACG	TTC	GGT	ACT	GGG	ACC	AGA	
	GTT	TCA	TTA	CTC	CTA	GGC	GAG	TGC	AAG	CCA	TGA	CCC	TGG	TCT	
							Spacer								
+1	L	E	I	K	R	A	A	A	A	R	A	P	E	M	
799	CTG	GAA	ATA	AAA	CGG	GCG	GCC	GCA	GCC	CGG	GCA	CCA	GAA	ATG	
	GAC	CTT	TAT	TTT	GCC	CGC	CGG	CGT	CGG	GCC	CGT	GGT	CTT	TAC	
+1	P	V	L	E	N	R	A	A	Q	G	D	I	T	A	
841	CCT	GTT	CTG	GAA	AAC	CGG	GCT	GCT	CAG	GGC	GAT	ATT	ACT	GCA	
	GGA	CAA	GAC	CTT	TTG	GCC	CGA	CGA	GTC	CCG	CTA	TAA	TGA	CGT	
+1	P	G	G	A	R	R	L	T	G	D	Q	T	A	A	
883	CCC	GGC	GGT	GCT	CGC	CGT	TTA	ACG	GGT	GAT	CAG	ACT	GCC	GCT	
	GGG	CCG	CCA	CGA	GCG	GCA	AAT	TGC	CCA	CTA	GTC	TGA	CGG	CGA	
+1	L	R	D	S	L	S	D	K	P	A	K	N	I	I	
925	CTG	CGT	GAT	TCT	CTT	AGC	GAT	AAA	CCT	GCA	AAA	AAT	ATT	ATT	
	GAC	GCA	CTA	AGA	GAA	TCG	CTA	TTT	GGA	CGT	TTT	TTA	TAA	TAA	

Fig. 26-2

```

+1  L  L  I  G  D  G  M  G  D  S  E  I  T  A
967  TTG CTG ATT GGC GAT GGG ATG GGG GAC TCG GAA ATT ACT GCC
    AAC GAC TAA CCG CTA CCC TAC CCC CTG AGC CTT TAA TGA CGG

+1  A  R  N  Y  A  E  G  A  G  G  F  F  K  G
1009  GCA CGT AAT TAT GCC GAA GGT GCG GGC GGC TTT TTT AAA GGT
    CGT GCA TTA ATA CGG CTT CCA CGC CCG CCG AAA AAA TTT CCA

+1  I  D  A  L  P  L  T  G  Q  Y  T  H  Y  A
1051  ATA GAT GCC TTA CCG CTT ACC GGG CAA TAC ACT CAC TAT GCG
    TAT CTA CGG AAT GGC GAA TGG CCC GTT ATG TGA GTG ATA CGC

+1  L  N  K  K  T  G  K  P  D  Y  V  T  D  S
1093  CTG AAT AAA AAA ACC GGC AAA CCG GAC TAC GTC ACC GAC TCG
    GAC TTA TTT TTT TGG CCG TTT GGC CTG ATG CAG TGG CTG AGC

+1  A  A  S  A  T  A  W  S  T  G  V  K  T  Y
1135  GCT GCA TCA GCA ACC GCC TGG TCA ACC GGT GTC AAA ACC TAT
    CGA CGT AGT CGT TGG CGG ACC AGT TGG CCA CAG TTT TGG ATA

+1  N  G  A  L  G  V  D  I  H  E  K  D  H  P
1177  AAC GGC GCG CTG GGC GTC GAT ATT CAC GAA AAA GAT CAC CCA
    TTG CCG CGC GAC CCG CAG CTA TAA GTG CTT TTT CTA GTG GGT

+1  T  I  L  E  M  A  K  A  A  G  L  A  T  G
1219  ACG ATT CTG GAA ATG GCA AAA GCC GCA GGT CTG GCG ACC GGT
    TGC TAA GAC CTT TAC CGT TTT CGG CGT CCA GAC CGC TGG CCA

+1  N  V  S  T  A  E  L  Q  D  A  T  P  A  A
1261  AAC GTT TCT ACC GCA GAG TTG CAG GAT GCC ACG CCC GCT GCG
    TTG CAA AGA TGG CGT CTC AAC GTC CTA CGG TGC GGG CGA CGC

+1  L  V  A  H  V  T  S  R  K  C  Y  G  P  S
1303  CTG GTG GCA CAT GTG ACC TCG CGC AAA TGC TAC GGT CCG AGC
    GAC CAC CGT GTA CAC TGG AGC GCG TTT ACG ATG CCA GGC TCG

+1  A  T  S  E  K  C  P  G  N  A  L  E  K  G
1345  GCG ACC AGT GAA AAA TGT CCG GGT AAC GCT CTG GAA AAA GGC
    CGC TGG TCA CTT TTT ACA GGC CCA TTG CGA GAC CTT TTT CCG

+1  G  K  G  S  I  T  E  Q  L  L  N  A  R  A
1387  GGA AAA GGA TCG ATT ACC GAA CAG CTG CTT AAC GCT CGT GCC
    CCT TTT CCT AGC TAA TGG CTT GTC GAC GAA TTG CGA GCA CGG

+1  D  V  T  L  G  G  G  A  K  T  F  A  E  T
1429  GAC GTT ACG CTT GGC GGC GGC GCA AAA ACC TTT GCT GAA ACG
    CTG CAA TGC GAA CCG CCG CCG CGT TTT TGG AAA CGA CTT TGC

```

Fig-26-3

```

+1  A  T  A  G  E  W  Q  G  K  T  L  R  E  Q
1471 GCA ACC GCT GGT GAA TGG CAG GGA AAA ACG CTG CGT GAA CAG
    CGT TGG CGA CCA CTT ACC GTC CCT TTT TGC GAC GCA CTT GTC

+1  A  Q  A  R  G  Y  Q  L  V  S  D  A  A  S
1513 GCA CAG GCG CGT GGT TAT CAG TTG GTG AGC GAT GCT GCC TCA
    CGT GTC CGC GCA CCA ATA GTC AAC CAC TCG CTA CGA CGG AGT

+1  L  N  S  V  T  E  A  N  Q  Q  K  P  L  L
1555 CTG AAT TCG GTG ACG GAA GCG AAT CAG CAA AAA CCC CTG CTT
    GAC TTA AGC CAC TGC CTT CGC TTA GTC GTT TTT GGG GAC GAA

+1  G  L  F  A  D  G  N  M  P  V  R  W  L  G
1597 GGC CTG TTT GCT GAC GGC AAT ATG CCA GTG CGC TGG CTA GGA
    CCG GAC AAA CGA CTG CCG TTA TAC GGT CAC GCG ACC GAT CCT

+1  P  K  A  T  Y  H  G  N  I  D  K  P  A  V
1639 CCG AAA GCA ACG TAC CAT GGC AAT ATC GAT AAG CCC GCA GTC
    GGC TTT CGT TGC ATG GTA CCG TTA TAG CTA TTC GGG CGT CAG

+1  T  C  T  P  N  P  Q  R  N  D  S  V  P  T
1681 ACC TGT ACG CCA AAT CCG CAA CGT AAT GAC AGT GTA CCA ACC
    TGG ACA TGC GGT TTA GGC GTT GCA TTA CTG TCA CAT GGT TGG

+1  L  A  Q  M  T  D  K  A  I  E  L  L  S  K
1723 CTG GCG CAG ATG ACC GAC AAA GCC ATT GAA TTG TTG AGT AAA
    GAC CGC GTC TAC TGG CTG TTT CGG TAA CTT AAC AAC TCA TTT

+1  N  E  K  G  F  F  L  Q  V  E  G  A  S  I
1765 AAT GAG AAA GGC TTT TTC CTG CAA GTT GAA GGT GCG TCA ATC
    TTA CTC TTT CCG AAA AAG GAC GTT CAA CTT CCA CGC AGT TAG

+1  D  K  Q  D  H  A  A  N  P  C  G  Q  I  G
1807 GAT AAA CAG GAT CAT GCT GCG AAT CCT TGT GGG CAA ATT GGC
    CTA TTT GTC CTA GTA CGA CGC TTA GGA ACA CCC GTT TAA CCG

+1  E  T  V  D  L  D  E  A  V  Q  R  A  L  E
1849 GAG ACG GTC GAT CTC GAT GAA GCC GTA CAA CGG GCG CTG GAA
    CTC TGC CAG CTA GAG CTA CTT CGG CAT GTT GCC CGC GAC CTT

+1  F  A  K  K  E  G  N  T  L  V  I  V  T  A
1891 TTC GCT AAA AAG GAG GGT AAC ACG CTG GTC ATA GTC ACC GCT
    AAG CGA TTT TTC CTC CCA TTG TGC GAC CAG TAT CAG TGG CGA

+1  D  H  A  H  A  S  Q  I  V  A  P  D  T  K
1933 GAT CAC GCC CAC GCC AGC CAG ATT GTT GCG CCG GAT ACC AAA
    CTA GTG CGG GTG CGG TCG GTC TAA CAA CGC GGC CTA TGG TTT

+1  A  P  G  L  T  Q  A  L  N  T  K  D  G  A
1975 GCT CCG GGC CTC ACC CAG GCG CTA AAT ACC AAA GAT GGC GCA
    CGA GGC CCG GAG TGG GTC CGC GAT TTA TGG TTT CTA CCG CGT

```

Fig. 26-4

	+1	V	M	V	M	S	Y	G	N	S	E	E	D	S	Q	
2017		GTG	ATG	GTG	ATG	AGT	TAC	GGG	AAC	TCC	GAA	GAG	GAT	TCA	CAA	
		CAC	TAC	CAC	TAC	TCA	ATG	CCC	TTG	AGG	CTT	CTC	CTA	AGT	GTT	
	+1	E	H	T	G	S	Q	L	R	I	A	A	Y	G	P	
2059		GAA	CAT	ACC	GGC	AGT	CAG	TTG	CGT	ATT	GCG	GCG	TAT	GGC	CCG	
		CTT	GTA	TGG	CCG	TCA	GTC	AAC	GCA	TAA	CGC	CGC	ATA	CCG	GGC	
	+1	H	A	A	N	V	V	G	L	T	D	Q	T	D	L	
2101		CAT	GCC	GCC	AAT	GTT	GTT	GGA	CTG	ACC	GAC	CAG	ACC	GAT	CTC	
		GTA	CGG	CGG	TTA	CAA	CAA	CCT	GAC	TGG	CTG	GTC	TGG	CTA	GAG	
	+1	F	Y	T	M	K	A	A	L	G	D	I	His tag			
2143		TTC	TAC	ACC	ATG	AAA	GCC	GCT	CTG	GGG	GAT	ATC	A	H	H	
		AAG	ATG	TGG	TAC	TTT	CGG	CGA	GAC	CCC	CTA	TAG	GCA	CAC	CAT	
													CGT	GTG	GTA	
	+1	H	H	H	H	*										
2185		CAC	CAT	CAC	CAT	TAA										
		GTG	GTA	GTG	GTA	ATT										

Fig. 26-5

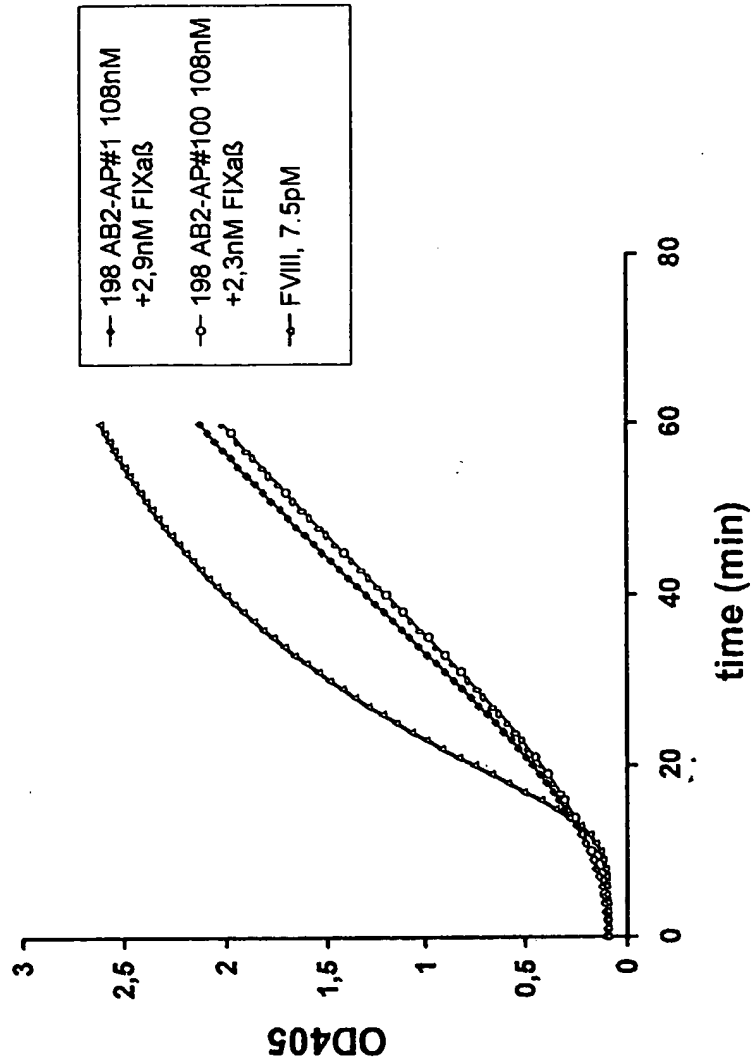


Fig. 27



**PelB-Leader**

+1 M K Y L L P T A A A G L L L L  
 1 ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC  
 TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT GAG

**VH**

+1 A A Q P A M A E V K L V E S G  
 46 GCG GCC CAG CCG GCC ATG GCG GAG GTG AAG CTG GTG GAG TCT GGG  
 CGC CGG GTC GGC CGG TAC CGC CTC CAC TTC GAC CAC CTC AGA CCC

+1 G G L V K P G G S L K L S C A  
 91 GGA GGC TTA GTG AAG CCT GGA GGG TCC CTG AAA CTC TCC TGT GCA  
 CCT CCG AAT CAC TTC GGA CCT CCC AGG GAC TTT GAG AGG ACA CGT

+1 A S G F T F S S Y T M S W V R  
 136 GCC TCT GGA TTC ACT TTC AGT AGC TAT ACC ATG TCT TGG GTT CGC  
 CGG AGA CCT AAG TGA AAG TCA TCG ATA TGG TAC AGA ACC CAA GCG

+1 Q T P E K R L E W V A T I S S  
 181 CAG ACT CCG GAG AAG AGG CTG GAG TGG GTC GCA ACC ATT AGT AGT  
 GTC TGA GGC CTC TTC TCC GAC CTC ACC CAG CGT TGG TAA TCA TCA

+1 G G S S T Y Y P D S V K G R F  
 226 GGN GGT AGT TCC ACC TAC TAT CCA GAC AGT GTG AAG GGC CGA TTC  
 CCN CCA TCA AGG TGG ATG ATA GGT CTG TCA CAC TTC CCG GCT AAG

+1 T I S R D N A K N T L Y L Q M  
 271 ACC ATC TCC AGA GAC AAT GCC AAG AAC ACC CTG TAC CTG CAA ATG  
 TGG TAG AGG TCT CTG TTA CGG TTC TTG TGG GAC ATG GAC GTT TAC

+1 S S L R S E D T A M Y Y C T R  
 316 AGC AGT CTG AGG TCT GAG GAC ACA GCC ATG TAT TAC TGT ACA AGA  
 TCG TCA GAC TCC AGA CTC CTG TGT CGG TAC ATA ATG ACA TGT TCT

+1 E G G G F T V N W Y F D V W G  
 361 GAG GGG GGT GGT TTC ACC GTC AAC TGG TAC TTC GAT GTC TGG GGC  
 CTC CCC CCA CCA AAG TGG CAG TTG ACC ATG AAG CTA CAG ACC CCG

**Linker**

+1 A G T S V T V S S G G G G S G  
 406 GCA GGA ACC TCA GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGT  
 CGT CCT TGG AGT CAG TGG CAG AGG AGT CCA CCT CCG CCA AGT CCA

**VL**

+1 G R A S G G G G S D I V L T Q  
 451 GGG CGC GCC TCT GGC GGT GGC GGA TCG GAC ATT GTG CTG ACA CAG  
 CCC GCG CGG AGA CCG CCA CCG CCT AGC CTG TAA CAC GAC TGT GTC

Fig. 2P-1

+1	X	P	A	S	L	A	V	S	L	G	Q	R	A	T	I	
496	TNT	CCA	GCT	TCT	TTG	GCT	GTG	TCT	CTA	GGG	CAG	AGG	GCC	ACC	ATA	
	ANA	GGT	CGA	AGA	AAC	CGA	CAC	AGA	GAT	CCC	GTC	TCC	CGG	TGG	TAT	
+1	S	C	R	A	S	E	S	V	D	S	Y	G	Y	N	F	
541	TCN	TGC	AGA	GCC	AGT	GAA	AGT	GTT	GAT	AGT	TAT	GGC	TAT	AAT	TTT	
	AGN	ACG	TCT	CGG	TCA	CTT	TCA	CAA	CTA	TCA	ATA	CCG	ATA	TTA	AAA	
+1	M	H	W	Y	Q	Q	I	P	G	Q	P	P	K	L	L	
586	ATG	CAC	TGG	TAT	CAG	CAG	ATA	CCA	GGA	CAG	CCA	CCC	AAA	CTC	CTC	
	TAC	GTG	ACC	ATA	GTC	GTC	TAT	GGT	CCT	GTC	GGT	GGG	TTT	GAG	GAG	
+1	I	Y	R	A	S	N	L	E	S	G	I	P	A	R	F	
631	ATC	TAT	CGT	GCA	TCC	AAC	CTA	GAG	TCT	GGG	ATC	CCT	GCC	AGG	TTC	
	TAG	ATA	GCA	CGT	AGG	TTG	GAT	CTC	AGA	CCC	TAG	GGA	CGG	TCC	AAG	
+1	S	G	S	G	S	R	T	D	F	T	L	T	I	N	P	
676	AGT	GGC	AGT	GGG	TCT	AGG	ACA	GAC	TTC	ACC	CTC	ACC	ATT	AAT	CCT	
	TCA	CCG	TCA	CCC	AGA	TCC	TGT	CTG	AAG	TGG	GAG	TGG	TAA	TTA	GGA	
+1	V	E	A	D	D	V	A	T	Y	Y	C	Q	Q	S	N	
721	GTG	GAG	GCT	GAT	GAT	GTT	GCA	ACC	TAT	TAC	TGT	CAG	CAA	AGT	AAT	
	CAC	CTC	CGA	CTA	CTA	CAA	CGT	TGG	ATA	ATG	ACA	GTC	GTT	TCA	TTA	
+1	E	D	P	L	T	F	G	T	G	T	R	L	E	I	K	
766	GAG	GAT	CCG	CTC	ACG	TTC	GGT	ACT	GGG	ACC	AGA	CTG	GAA	ATA	AAA	
	CTC	CTA	GGC	GAG	TGC	AAG	CCA	TGA	CCC	TGG	TCT	GAC	CTT	TAT	TTT	
	Spacer				Hinge				Helix							
+1	R	A	A	A	P	K	P	S	T	P	P	G	S	S	R	
811	CGG	GCG	GCC	GCA	CCG	AAG	CCT	TCC	ACT	CCG	CCC	GGG	TCT	TCC	CGT	
	GCC	CGC	CGG	CGT	GGC	TTC	GGA	AGG	TGA	GGC	GGG	CCC	AGA	AGG	GCA	
+1	M	K	Q	L	E	D	K	V	E	E	L	L	S	K	N	
856	ATG	AAA	CAG	CTG	GAA	GAC	AAA	GTA	GAG	GAG	CTC	CTT	AGC	AAG	AAC	
	TAC	TTT	GTC	GAC	CTT	CTG	TTT	CAT	CTC	CTC	GAG	GAA	TCG	TTC	TTG	
+1	Y	H	L	E	N	E	V	A	R	L	K	K	L	V	G	
901	TAC	CAT	CTA	GAA	AAC	GAG	GTA	GCT	CGT	CTG	AAA	AAG	CTT	GTT	GGT	
	ATG	GTA	GAT	CTT	TTG	CTC	CAT	CGA	GCA	GAC	TTT	TTC	GAA	CAA	CCA	
	Spacer				His-tag											
+1	E	R	G	G	H	H	H	H	H	H	*					
946	GAA	CGT	GGT	GGT	CAC	CAT	CAC	CAT	CAC	CAT	TAA					
	CTT	GCA	CCA	CCA	GTG	GTA	GTG	GTA	GTG	GTA	ATT					

Fig. 28-2

+1	M	K	Y	L	L	P	T	A	A	A	G	L	L	L
1	ATG	AAA	TAC	CTA	TTG	CCT	ACG	GCA	GCC	GCT	GGA	TTG	TTA	TTA
	TAC	TTT	ATG	GAT	AAC	GGA	TGC	CGT	CGG	CGA	CCT	AAC	AAT	AAT

VH

	+1	A	M	D	Y	W	G	Q	G	T	S	V	T	V	S
379		GCT	ATG	GAC	TAC	TGG	GGT	CAA	GGA	ACC	TCA	GTC	ACC	GTC	TCC
		CGA	TAC	CTG	ATG	ACC	CCA	GTT	CCT	TGG	AGT	CAG	TGG	CAG	AGG

## Linker

·VL

46/61

```

+1  V  S  L  G  Q  R  A  T  I  S  C  R  A  S
505  GTA TCT CTG GGG CAG AGG GCC ACC ATC TCA TGC AGG GCC AGC
    CAT AGA GAC CCC GTC TCC CGG TGG TAG AGT ACG TCC CGG TCG

+1  K  S  V  S  T  S  G  Y  S  Y  M  H  W  Y
547  AAA AGT GTC AGT ACA TCT GGC TAT AGT TAT ATG CAC TGG TAC
    TTT TCA CAG TCA TGT AGA CCG ATA TCA ATA TAC GTG ACC ATG

+1  Q  Q  K  P  G  Q  P  P  K  L  L  I  Y  L
589  CAA CAG AAA CCA GGA CAG CCA CCC AAA CTC CTC ATC TAT CTT
    GTT GTC TTT GGT CCT GTC GGT GGG TTT GAG GAG TAG ATA GAA

+1  A  S  N  L  E  S  G  V  P  A  R  F  S  G
631  GCA TCC AAC CTA GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC
    CGT AGG TTG GAT CTT AGA CCC CAG GGA CGG TCC AAG TCA CCG

+1  S  G  S  G  T  D  F  T  L  N  I  H  P  V
673  AGT GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG
    TCA CCC AGA CCC TGT CTG AAG TGG GAG TTG TAG GTA GGA CAC

+1  E  E  E  D  A  A  T  Y  Y  C  Q  H  S  R
715  GAG GAG GAG GAT GCT GCA ACC TAT TAC TGT CAG CAC AGT AGG
    CTC CTC CTC CTA CGA CGT TGG ATA ATG ACA GTC GTG TCA TCC

+1  E  L  P  R  T  F  G  G  G  T  K  L  E  I
757  GAG CTT CCT CGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC
    CTC GAA GGA GCC TGC AAG CCA CCT CCG TGG TTC GAC CTT TAG

+1  K  R  |  Spacer  |  Alkaline phosphatase
799  AAA CGG | GCG GCC GCA GCC | CGG GCA CCA GAA ATG CCT GTT CTG
    TTT GCC | CGC CGG CGT CGG | GCC CGT GGT CTT TAC GGA CAA GAC

+1  E  N  R  A  A  Q  G  D  I  T  A  P  G  G
841  GAA AAC CGG GCT GCT CAG GGC GAT ATT ACT GCA CCC GGC GGT
    CTT TTG GCC CGA CGA GTC CCG CTA TAA TGA CGT GGG CCG CCA

+1  A  R  R  L  T  G  D  Q  T  A  A  L  R  D
883  GCT CGC CGT TTA ACG GGT GAT CAG ACT GCC GCT CTG CGT GAT
    CGA GCG GCA AAT TGC CCA CTA GTC TGA CGG CGA GAC GCA CTA

+1  S  L  S  D  K  P  A  K  N  I  I  L  L  I
925  TCT CTT AGC GAT AAA CCT GCA AAA AAT ATT ATT TTG CTG ATT
    AGA GAA TCG CTA TTT GGA CGT TTT TTA TAA TAA AAC GAC TAA

+1  G  D  G  M  G  D  S  E  I  T  A  A  R  N
967  GGC GAT GGG ATG GGG GAC TCG GAA ATT ACT GCC GCA CGT AAT
    CCG CTA CCC TAC CCC CTG AGC CTT TAA TGA CGG CGT GCA TTA

```

Fig. 29-2

```

+1  Y  A  E  G  A  G  G  F  F  K  G  I  D  A
1009  TAT GCC GAA GGT GCG GGC GGC TTT TTT AAA GGT ATA GAT GCC
      ATA CGG CTT CCA CGC CCG CCG AAA AAA TTT CCA TAT CTA CGG

+1  L  P  L  T  G  Q  Y  T  H  Y  A  L  N  K
1051  TTA CCG CTT ACC GGG CAA TAC ACT CAC TAT GCG CTG AAT AAA
      AAT GGC GAA TGG CCC GTT ATG TGA GTG ATA CGC GAC TTA TTT

+1  K  T  G  K  P  D  Y  V  T  D  S  A  A  S
1093  AAA ACC GGC AAA CCG GAC TAC GTC ACC GAC TCG GCT GCA TCA
      TTT TGG CCG TTT GGC CTG ATG CAG TGG CTG AGC CGA CGT AGT

+1  A  T  A  W  S  T  G  V  K  T  Y  N  G  A
1135  GCA ACC GCC TGG TCA ACC GGT GTC AAA ACC TAT AAC GGC GCG
      CGT TGG CGG ACC AGT TGG CCA CAG TTT TGG ATA TTG CCG CGC

+1  L  G  V  D  I  H  E  K  D  H  P  T  I  L
1177  CTG GGC GTC GAT ATT CAC GAA AAA GAT CAC CCA ACG ATT CTG
      GAC CCG CAG CTA TAA GTG CTT TTT CTA GTG GGT TGC TAA GAC

+1  E  M  A  K  A  A  G  L  A  T  G  N  V  S
1219  GAA ATG GCA AAA GCC GCA GGT CTG GCG ACC GGT AAC GTT TCT
      CTT TAC CGT TTT CGG CGT CCA GAC CGC TGG CCA TTG CAA AGA

+1  T  A  E  L  Q  D  A  T  P  A  A  L  V  A
1261  ACC GCA GAG TTG CAG GAT GCC ACG CCC GCT GCG CTG GTG GCA
      TGG CGT CTC AAC GTC CTA CGG TGC GGG CGA CGC GAC CAC CGT

+1  H  V  T  S  R  K  C  Y  G  P  S  A  T  S
1303  CAT GTG ACC TCG CGC AAA TGC TAC GGT CCG AGC GCG ACC AGT
      GTA CAC TGG AGC GCG TTT ACG ATG CCA GGC TCG CGC TGG TCA

+1  E  K  C  P  G  N  A  L  E  K  G  G  K  G
1345  GAA AAA TGT CCG GGT AAC GCT CTG GAA AAA GGC GGA AAA GGA
      CTT TTT ACA GGC CCA TTG CGA GAC CTT TTT CCG CCT TTT CCT

+1  S  I  T  E  Q  L  L  N  A  R  A  D  V  T
1387  TCG ATT ACC GAA CAG CTG CTT AAC GCT CGT GCC GAC GTT ACG
      AGC TAA TGG CTT GTC GAC GAA TTG CGA GCA CGG CTG CAA TGC

+1  L  G  G  G  A  K  T  F  A  E  T  A  T  A
1429  CTT GGC GGC GGC GCA AAA ACC TTT GCT GAA ACG GCA ACC GCT
      GAA CCG CCG CCG CGT TTT TGG AAA CGA CTT TGC CGT TGG CGA

+1  G  E  W  Q  G  K  T  L  R  E  Q  A  Q  A
1471  GGT GAA TGG CAG GGA AAA ACG CTG CGT GAA CAG GCA CAG GCG
      CCA CTT ACC GTC CCT TTT TGC GAC GCA CTT GTC CGT GTC CGC

```

Fig. 29-3

+1	R	G	Y	Q	L	V	S	D	A	A	S	L	N	S
1513	CGT	GGT	TAT	CAG	TTG	GTG	AGC	GAT	GCT	GCC	TCA	CTG	AAT	TCG
	GCA	CCA	ATA	GTC	AAC	CAC	TCG	CTA	CGA	CGG	AGT	GAC	TTA	AGC
+1	V	T	E	A	N	Q	Q	K	P	L	L	G	L	F
1555	GTG	ACG	GAA	GCG	AAT	CAG	CAA	AAA	CCC	CTG	CTT	GGC	CTG	TTT
	CAC	TGC	CTT	CGC	TTA	GTC	GTT	TTT	GGG	GAC	GAA	CCG	GAC	AAA
+1	A	D	G	N	M	P	V	R	W	L	G	P	K	A
1597	GCT	GAC	GGC	AAT	ATG	CCA	GTG	CGC	TGG	CTA	GGA	CCG	AAA	GCA
	CGA	CTG	CCG	TTA	TAC	GGT	CAC	GCG	ACC	GAT	CCT	GGC	TTT	CGT
+1	T	Y	H	G	N	I	D	K	P	A	V	T	C	T
1639	ACG	TAC	CAT	GGC	AAT	ATC	GAT	AAG	CCC	GCA	GTC	ACC	TGT	ACG
	TGC	ATG	GTA	CCG	TTA	TAG	CTA	TTC	GGG	CGT	CAG	TGG	ACA	TGC
+1	P	N	P	Q	R	N	D	S	V	P	T	L	A	Q
1681	CCA	AAT	CCG	CAA	CGT	AAT	GAC	AGT	GTA	CCA	ACC	CTG	GCG	CAG
	GGT	TTA	GGC	GTT	GCA	TTA	CTG	TCA	CAT	GGT	TGG	GAC	CGC	GTC
+1	M	T	D	K	A	I	E	L	L	S	K	N	E	K
1723	ATG	ACC	GAC	AAA	GCC	ATT	GAA	TTG	TTG	AGT	AAA	AAT	GAG	AAA
	TAC	TGG	CTG	TTT	CGG	TAA	CTT	AAC	AAC	TCA	TTT	TTA	CTC	TTT
+1	G	F	F	L	Q	V	E	G	A	S	I	D	K	Q
1765	GGC	TTT	TTC	CTG	CAA	GTT	GAA	GGT	GCG	TCA	ATC	GAT	AAA	CAG
	CCG	AAA	AAG	GAC	GTT	CAA	CTT	CCA	CGC	AGT	TAG	CTA	TTT	GTC
+1	D	H	A	A	N	P	C	G	Q	I	G	E	T	V
1807	GAT	CAT	GCT	GCG	AAT	CCT	TGT	GGG	CAA	ATT	GGC	GAG	ACG	GTC
	CTA	GTA	CGA	CGC	TTA	GGA	ACA	CCC	GTT	TAA	CCG	CTC	TGC	CAG
+1	D	L	D	E	A	V	Q	R	A	L	E	F	A	K
1849	GAT	CTC	GAT	GAA	GCC	GTA	CAA	CGG	GCG	CTG	GAA	TTC	GCT	AAA
	CTA	GAG	CTA	CTT	CGG	CAT	GTT	GCC	CGC	GAC	CTT	AAG	CGA	TTT
+1	K	E	G	N	T	L	V	I	V	T	A	D	H	A
1891	AAG	GAG	GGT	AAC	ACG	CTG	GTC	ATA	GTC	ACC	GCT	GAT	CAC	GCC
	TTC	CTC	CCA	TTG	TGC	GAC	CAG	TAT	CAG	TGG	CGA	CTA	GTG	CGG
+1	H	A	S	Q	I	V	A	P	D	T	K	A	P	G
1933	CAC	GCC	AGC	CAG	ATT	GTT	GCG	CCG	GAT	ACC	AAA	GCT	CCG	GGC
	GTG	CGG	TCG	GTC	TAA	CAA	CGC	GGC	CTA	TGG	TTT	CGA	GGC	CCG
+1	L	T	Q	A	L	N	T	K	D	G	A	V	M	V
1975	CTC	ACC	CAG	GCG	CTA	AAT	ACC	AAA	GAT	GGC	GCA	GTG	ATG	GTG
	GAG	TGG	GTC	CGC	GAT	TTA	TGG	TTT	CTA	CCG	CGT	CAC	TAC	CAC

Fig. 29-4

```

+1  M   S   Y   G   N   S   E   E   D   S   Q   E   H   T
2017 ATG AGT TAC GGG AAC TCC GAA GAG GAT TCA CAA GAA CAT ACC
      TAC TCA ATG CCC TTG AGG CTT CTC CTA AGT GTT CTT GTA TGG

+1  G   S   Q   L   R   I   A   A   Y   G   P   H   A   A
2059 GGC AGT CAG TTG CGT ATT GCG GCG TAT GGC CCG CAT GCC GCC
      CCG TCA GTC AAC GCA TAA CGC CGC ATA CCG GGC GTA CGG CGG

+1  N   V   V   G   L   T   D   Q   T   D   L   F   Y   T
2101 AAT GTT GTT GGA CTG ACC GAC CAG ACC GAT CTC TTC TAC ACC
      TTA CAA CAA CCT GAC TGG CTG GTC TGG CTA GAG AAG ATG TGG

+1  M   K   A   A   L   G   D   I   A   |   His tag
2143 ATG AAA GCC GCT CTG GGG GAT ATC GCA CAC CAT CAC CAT CAC
      TAC TTT CGG CGA GAC CCC CTA TAG CGT GTG GTA GTG GTA GTG

+1  H   *
2185 CAT TAA
      GTA ATT

```

Fig. 29-5

PelB-Leader

```

+1  M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L
1   ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC
    TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT GAG

```

VH

```

+1  A   A   Q   P   A   M   A   E   V   Q   L   Q   Q   S   G
46  GCG GCC CAG CCG GCC ATG GCG GAG GTT CAG CTT CAG CAG TCT GGA
    CGC CGG GTC GGC CGG TAC CGC CTC CAA GTC GAA GTC GTC AGA CCT

```

```

+1  P   E   L   V   K   P   G   A   S   V   K   I   S   C   K
91  CCT GAG CTG GTG AAG CCC GGG GCC TCA GTG AAG ATT TCC TGC AAA
    GGA CTC GAC CAC TTC GGG CCC CGG AGT CAC TTC TAA AGG ACG TTT

```

```

+1  A   S   G   Y   A   F   S   S   S   W   M   N   W   V   K
136 GCT TCT GGC TAC GCA TTC AGT AGC TCT TGG ATG AAC TGG GTG AAG
    CGA AGA CCG ATG CGT AAG TCA TCG AGA ACC TAC TTG ACC CAC TTC

```

```

+1  Q   R   P   G   Q   G   L   E   W   I   G   R   I   Y   P
181 CAG AGG CCT GGA CAG GGT CTT GAG TGG ATT GGA CGG ATT TAT CCT
    GTC TCC GGA CCT GTC CCA GAA CTC ACC TAA CCT GCC TAA ATA GGA

```

```

+1  G   N   G   D   T   N   Y   N   G   K   F   K   G   K   A
226 GGA AAT GGA GAT ACT AAC TAC AAT GGG AAG TTC AAG GGC AAG GCC
    CCT TTA CCT CTA TGA TTG ATG TTA CCC TTC AAG TTC CCG TTC CGG

```

```

+1  T   L   T   A   D   K   S   S   S   T   A   Y   M   Q   L
271 ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC
    TGT GAC TGA CGT CTG TTT AGG AGG TCG TGT CGG ATG TAC GTC GAG

```

```

+1  S   S   L   T   S   V   D   S   A   V   Y   F   C   A   D
316 AGC AGC CTG ACC TCT GTG GAC TCT GCG GTC TAT TTC TGT GCA GAT
    TCG TCG GAC TGG AGA CAC CTG AGA CGC CAG ATA AAG ACA CGT CTA

```

```

+1  G   N   V   Y   Y   Y   A   M   D   Y   W   G   Q   G   T
361 GGT AAC GTA TAT TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC
    CCA TTG CAT ATA ATG ATA CGA TAC CTG ATG ACC CCA GTT CCT TGG

```

Linker

```

+1  S   V   T   V   S   S   G   G   G   G   S   G   G   R   A
406 TCA GTC ACC GTC TCC TCA GGT GGA GGC GGT TCA GGT GGG CGC GCC
    AGT CAG TGG CAG AGG AGT CCA CCT CCG CCA AGT CCA CCC GCG CGG

```

Fig. 30-1



Fig. 30-2

Spacer		His-tag						
+1	G	H	H	H	H	H	H	*
946	GGT	CAC	CAT	CAC	CAT	CAC	CAT	TAA
	CCA	GTG	GTA	GTG	GTA	GTG	GTA	ATT

Fig. 30-3

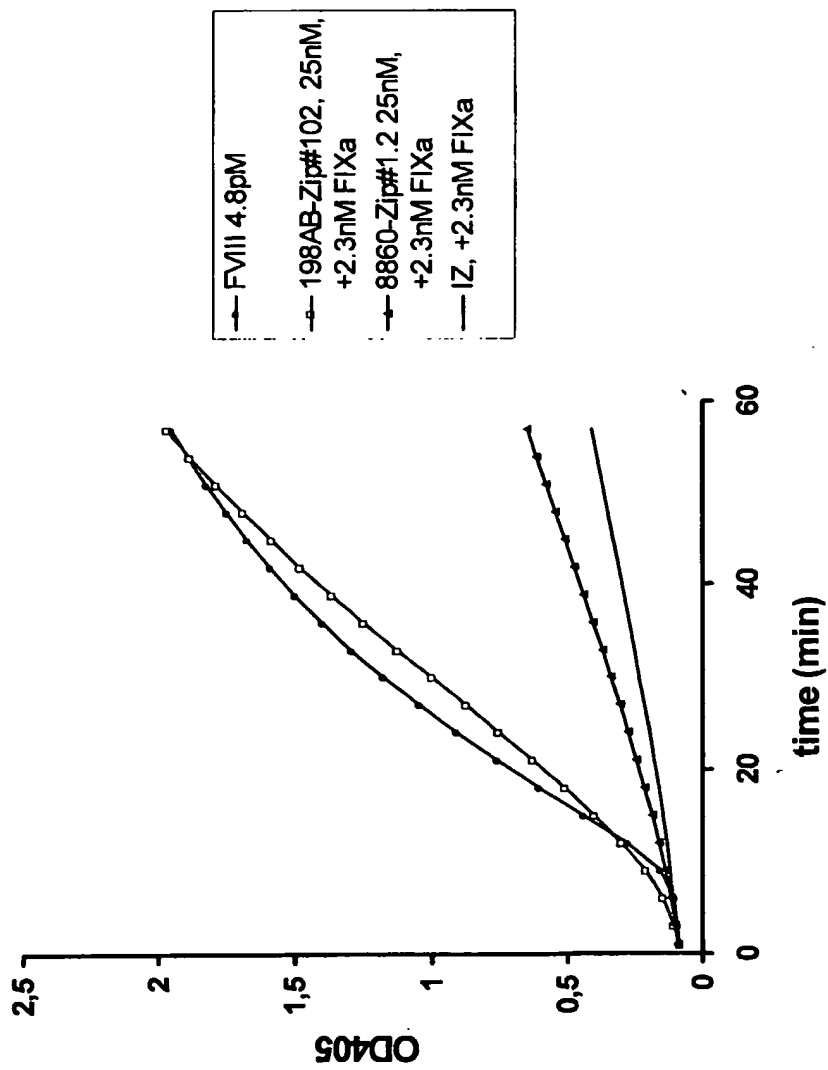


Fig. 31

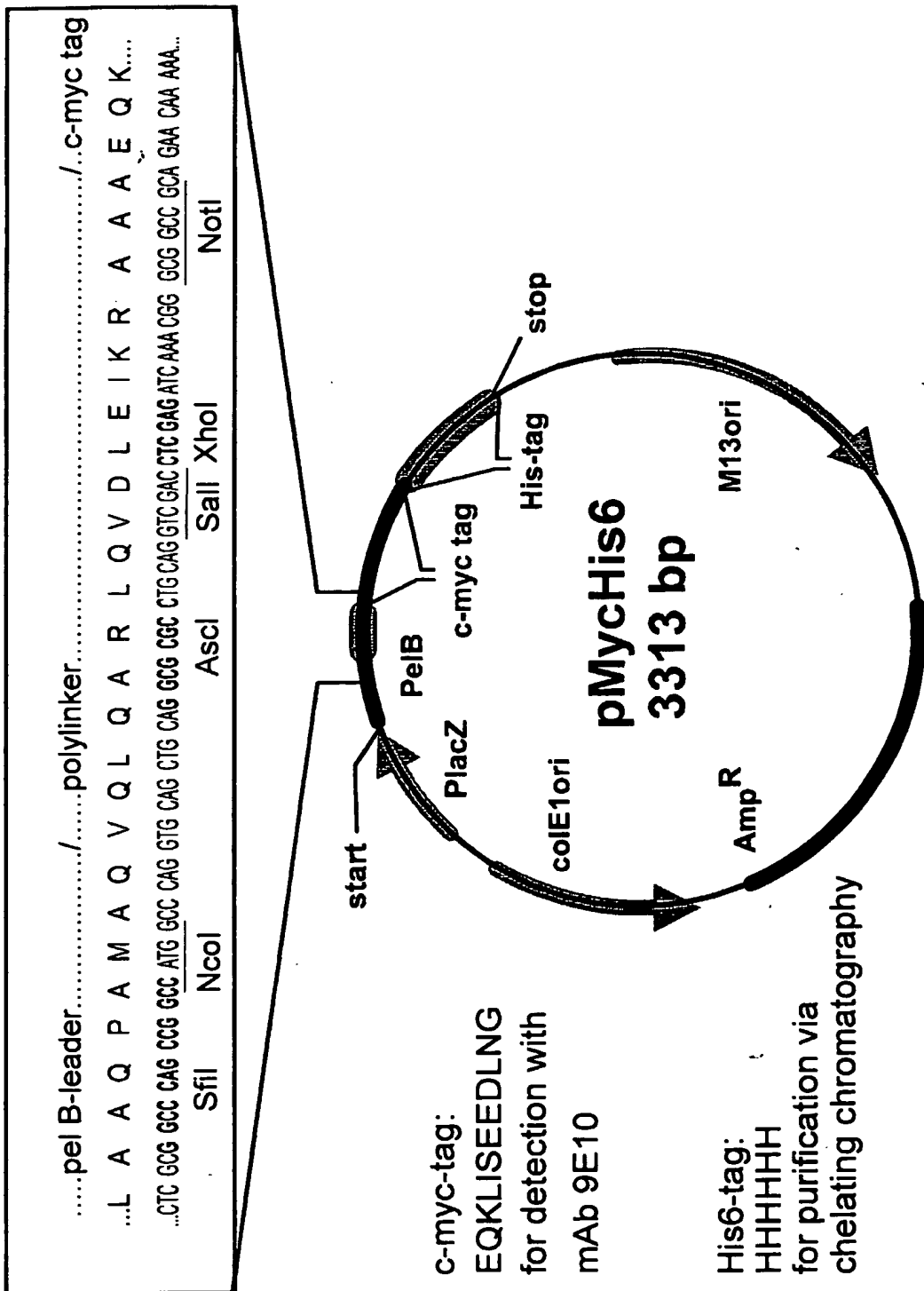


Fig. 32

*HindIII*  
~~~~~

2206 CAG GAA ACA GCT ATG ACC ATG ATT ACG CCA AGC TTC CAT GAA AAT  
GTC CTT TGT CGA TAC TGG TAC TAA TGC GGT TCG AAG GTA CTT TTA

PelB-Leader  
M K Y L L P T

2251 TCT ATT TCA AGG AGA CAG TCA TAA TGA AAT ACC TAT TGC CTA CGG  
AGA TAA AGT TCC TCT GTC AGT ATT ACT TTA TGG ATA ACG GAT GCC

A A A G L L L L A A Q P A M A

*SfiI*  
~~~~~

2296 CAG CCG CTG GAT TGT TAT TAC TCG CGG CCC AGC CGG CCA TGG CCC  
GTC GGC GAC CTA ACA ATA ATG AGC GCC GGG TCG GCC GGT ACC GGG

Polylinker  
Q V Q L Q A R L Q V D L E I K

*AscI*  
~~~~~

2341 AGG TGC AGC TGC AGG CGC GCC TGC AGG TCG ACC TCG AGA TCA AAC  
TCC ACG TCG ACG TCC GCG CGG ACG TCC AGC TGG AGC TCT AGT TTG

Spacer      Myc-tag  
R | A A A | E Q K L I S E E D L N  
NotI

2386 GGG CGG CCG CAG AAC AAA AAC TCA TCT CAG AAG AGG ATC TGA ATG  
CCC GCC GGC GTC TTG TTT TTG AGT AGA GTC TTC TCC TAG ACT TAC

Spacer      His tag  
G | A A | H H H H H H \* \*  
EcoRI

2431 GGG CGG CAC ATC ACC ATC ACC ATC ACT AAT AAG AAT TCA CTG GCC  
CCC GCC GTG TAG TGG TAG TGG TAG TGA TTA TTC TTA AGT GAC CGG

Fig. 33

PelB-leader

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +1 | M   | K   | Y   | L   | L   | P   | T   | A   | A   | A   | G   | L   | L   | L   | L   |
| 1  | ATG | AAA | TAC | CTA | TTG | CCT | ACG | GCA | GCC | GCT | GGA | TTG | TTA | TTA | CTC |
|    | TAC | TTT | ATG | GAT | AAC | GGA | TGC | CGT | CGG | CGA | CCT | AAC | AAT | AAT | GAG |

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|
|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |
| +1 | A   | A   | Q   | P   | A   | M   | A   | VH  | E   | V   | K   | L   | V   | E   | S   | G |
| 46 | GCG | GCC | CAG | CCG | GCC | ATG | GCC | GAG | GTG | AAG | CTG | GTG | GAG | TCT | GGG |   |
|    | CGC | CGG | GTC | GGC | CGG | TAC | CGG | CTC | CAC | TTC | GAC | CAC | CTC | AGA | CCC |   |

|    |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +1 | G   | G   | L   | V   | K   | P   | G   | G   | S   | L   | K   | L   | S   | C   | A   |
| 91 | GGA | GGC | TTA | GTG | AAG | CCT | GGA | GGG | TCC | CTG | AAA | CTC | TCC | TGT | GCA |
|    | CCT | CCG | AAT | CAC | TTC | GGA | CCT | CCC | AGG | GAC | TTT | GAG | AGG | ACA | CGT |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +1  | A   | S   | G   | F   | T   | F   | S   | S   | Y   | T   | M   | S   | W   | V   | R   |
| 136 | GCC | TCT | GGA | TTC | ACT | TTC | AGT | AGC | TAT | ACC | ATG | TCT | TGG | GTT | CGC |
|     | CGG | AGA | CCT | AAG | TGA | AAG | TCA | TCG | ATA | TGG | TAC | AGA | ACC | CAA | GCG |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +1  | Q   | T   | P   | E   | K   | R   | L   | E   | W   | V   | A   | T   | I   | S   | S   |
| 181 | CAG | ACT | CCG | GAG | AAG | AGG | CTG | GAG | TGG | GTC | GCA | ACC | ATT | AGT | AGT |
|     | GTC | TGA | GGC | CTC | TTC | TCC | GAC | CTC | ACC | CAG | CGT | TGG | TAA | TCA | TCA |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +1  | G   | G   | S   | S   | T   | Y   | Y   | P   | D   | S   | V   | K   | G   | R   | F   |
| 226 | GGN | GGT | AGT | TCC | ACC | TAC | TAT | CCA | GAC | AGT | GTG | AAG | GGC | CGA | TTC |
|     | CCN | CCA | TCA | AGG | TGG | ATG | ATA | GGT | CTG | TCA | CAC | TTC | CCG | GCT | AAG |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +1  | T   | I   | S   | R   | D   | N   | A   | K   | N   | T   | L   | Y   | L   | Q   | M   |
| 271 | ACC | ATC | TCC | AGA | GAC | AAT | GCC | AAG | AAC | ACC | CTG | TAC | CTG | CAA | ATG |
|     | TGG | TAG | AGG | TCT | CTG | TTA | CGG | TTC | TTG | TGG | GAC | ATG | GAC | GTT | TAC |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +1  | S   | S   | L   | R   | S   | E   | D   | T   | A   | M   | Y   | Y   | C   | T   | R   |
| 316 | AGC | AGT | CTG | AGG | TCT | GAG | GAC | ACA | GCC | ATG | TAT | TAC | TGT | ACA | AGA |
|     | TCG | TCA | GAC | TCC | AGA | CTC | CTG | TGT | CGG | TAC | ATA | ATG | ACA | TGT | TCT |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| +1  | E   | G   | G   | G   | F   | T   | V   | N   | W   | Y   | F   | D   | V   | W   | G   |
| 361 | GAG | GGG | GGT | GGT | TTC | ACC | GTC | AAC | TGG | TAC | TTC | GAT | GTC | TGG | GGC |
|     | CTC | CCC | CCA | CCA | AAG | TGG | CAG | TTG | ACC | ATG | AAG | CTA | CAG | ACC | CCG |

|     |     |     |     |     |     |     |     |     |     |        |     |     |     |     |     |   |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|--------|-----|-----|-----|-----|-----|---|
|     |     |     |     |     |     |     |     |     |     |        |     |     |     |     |     |   |
| +1  | A   | G   | T   | S   | V   | T   | V   | S   | S   | Leader | G   | G   | G   | G   | S   | G |
| 406 | GCA | GGA | ACC | TCA | GTC | ACC | GTC | TCC | TCA | GGT    | GGA | GGC | GGT | TCA | GGT |   |
|     | CGT | CCT | TGG | AGT | CAG | TGG | CAG | AGG | AGT | CCA    | CCT | CCG | CCA | AGT | CCA |   |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|---|
|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |   |
| +1  | G   | R   | A   | S   | G   | G   | G   | G   | S   | VK  | D   | I   | V   | L   | T   | Q |
| 451 | GGG | CGC | GCC | TCT | GGC | GGT | GGC | GGA | TCG | GAC | ATT | GTG | CTG | ACA | CAG |   |

Fig. 34-1

```

CCC GCG CGG AGA CCG CCA CCG CCT AGC CTG TAA CAC GAC TGT GTC

+1  S  P  A  S  L  A  V  S  L  G  Q  R  A  T  I
496 TCT CCA GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG GCC ACC ATA
    AGA GGT CGA AGA AAC CGA CAC AGA GAT CCC GTC TCC CGG TGG TAT

+1  S  C  R  A  S  E  S  V  D  S  Y  G  Y  N  F
541 TCC TGC AGA GCC AGT GAA AGT GTT GAT AGT TAT GGC TAT AAT TTT
    AGG ACG TCT CGG TCA CTT TCA CAA CTA TCA ATA CCG ATA TTA AAA

+1  M  H  W  Y  Q  Q  I  P  G  Q  P  P  K  L  L
586 ATG CAC TGG TAT CAG CAG ATA CCA GGA CAG CCA CCC AAA CTC CTC
    TAC GTG ACC ATA GTC GTC TAT GGT CCT GTC GGT GGG TTT GAG GAG

+1  I  Y  R  A  S  N  L  E  S  G  I  P  A  R  F
631 ATC TAT CGT GCA TCC AAC CTA GAG TCT GGG ATC CCT GCC AGG TTC
    TAG ATA GCA CGT AGG TTG GAT CTC AGA CCC TAG GGA CGG TCC AAG

+1  S  G  S  G  S  R  T  D  F  T  L  T  I  N  P
676 AGT GGC AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC ATT AAT CCT
    TCA CCG TCA CCC AGA TCC TGT CTG AAG TGG GAG TGG TAA TTA GGA

+1  V  E  A  D  D  V  A  T  Y  Y  C  Q  Q  S  N
721 GTG GAG GCT GAT GAT GTT GCA ACC TAT TAC TGT CAG CAA AGT AAT
    CAC CTC CGA CTA CTA CAA CGT TGG ATA ATG ACA GTC GTT TCA TTA

+1  E  D  P  L  T  F  G  T  G  T  R  L  E  I  K
766 GAG GAT CCG CTC ACG TTC GGT ACT GGG ACC AGA CTG GAA ATA AAA
    CTC CTA GGC GAG TGC AAG CCA TGA CCC TGG TCT GAC CTT TAT TTT

+1  R  |  Spacer  |  Myc-tag
    CGG | A  A  A  | E  Q  K  L  I  S  E  E  D  L  N
    GCC | GCG GCC GCA | GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT
        | CGC CGG CGT | CTT GTT TTT GAG TAG AGT CTT CTC CTA GAC TTA

+1  G  |  Spacer  |  His tag
    GGG | A  A  | H  H  H  H  H  H  *  *
    CCC | GCG GCA | CAT CAC CAT CAC CAT CAC TAA TAA
        | CGC CGT | GTA GTG GTA GTG GTA GTG ATT ATT

```

Fig. 34-2

PelB-leader

```

+1  M   K   Y   L   L   P   T   A   A   A   G   L   L   L   L
1   ATG AAA TAC CTA TTG CCT ACG GCA GCC GCT GGA TTG TTA TTA CTC
    TAC TTT ATG GAT AAC GGA TGC CGT CGG CGA CCT AAC AAT AAT GAG

      ,
+1  A   A   Q   P   A   M   A   |   VH
46  GCG GCC CAG CCG GCC ATG GCC | GAG GTT CAG CTT CAG CAG TCT GGA
    CGC CGG GTC GGC CGG TAC CGG | CTC CAA GTC GAA GTC GTC AGA CCT

+1  P   E   L   V   K   P   G   A   S   V   K   I   S   C   K
91  CCT GAG CTG GTG AAG CCC GGG GCC TCA GTG AAG ATT TCC TGC AAA
    GGA CTC GAC CAC TTC GGG CCC CGG AGT CAC TTC TAA AGG ACG TTT

+1  A   S   G   Y   A   F   S   S   S   W   M   N   W   V   K
136 GCT TCT GGC TAC GCA TTC AGT AGC TCT TGG ATG AAC TGG GTG AAG
    CGA AGA CCG ATG CGT AAG TCA TCG AGA ACC TAC TTG ACC CAC TTC

+1  Q   R   P   G   Q   G   L   E   W   I   G   R   I   Y   P
181 CAG AGG CCT GGA CAG GGT CTT GAG TGG ATT GGA CGG ATT TAT CCT
    GTC TCC GGA CCT GTC CCA GAA CTC ACC TAA CCT GCC TAA ATA GGA

+1  G   N   G   D   T   N   Y   N   G   K   F   K   G   K   A
226 GGA AAT GGA GAT ACT AAC TAC AAT GGG AAG TTC AAG GGC AAG GCC
    CCT TTA CCT CTA TGA TTG ATG TTA CCC TTC AAG TTC CCG TTC CGG

+1  T   L   T   A   D   K   S   S   S   T   A   Y   M   Q   L
271 ACA CTG ACT GCA GAC AAA TCC TCC AGC ACA GCC TAC ATG CAG CTC
    TGT GAC TGA CGT CTG TTT AGG AGG TCG TGT CGG ATG TAC GTC GAG

+1  S   S   L   T   S   V   D   S   A   V   Y   F   C   A   D
316 AGC AGC CTG ACC TCT GTG GAC TCT GCG GTC TAT TTC TGT GCA GAT
    TCG TCG GAC TGG AGA CAC CTG AGA CGC CAG ATA AAG ACA CGT CTA

+1  G   N   V   Y   Y   Y   A   M   D   Y   W   G   Q   G   T
361 GGT AAC GTA TAT TAC TAT GCT ATG GAC TAC TGG GGT CAA GGA ACC
    CCA TTG CAT ATA ATG ATA CGA TAC CTG ATG ACC CCA GTT CCT TGG

      |   Leader
+1  S   V   T   V   S   S   |   G   G   G   G   S   G   G   R   A
406 TCA GTC ACC GTC TCC TCA | GGT GGA GGC GGT TCA GGT GGG CGC GCC
    AGT CAG TGG CAG AGG AGT | CCA CCT CCG CCA AGT CCA CCC GCG CGG

      |   VL
+1  S   G   G   G   G   S   |   Q   I   V   L   T   Q   S   P   A
451 TCT GGC GGT GGC GGA TCG | CAA ATT GTT CTC ACC CAG TCT CCT GCT
    AGA CCG CCA CCG CCT AGC | GTT TAA CAA GAG TGG GTC AGA GGA CGA

```

Fig. 35-1



```

+1  S   L   A   V   S   L   G   Q   R   A   T   I   S   C   R
496  TCC TTA GCT GTA TCT CTG GGG CAG AGG GCC ACC ATC TCA TGC AGG
    AGG AAT CGA CAT AGA GAC CCC GTC TCC CGG TGG TAG AGT ACG TCC

+1  A   S   K   S   V   S   T   S   G   Y   S   Y   M   H   W
541  GCC AGC AAA AGT GTC AGT ACA TCT GGC TAT AGT TAT ATG CAC TGG
    CGG TCG TTT TCA CAG TCA TGT AGA CCG ATA TCA ATA TAC GTG ACC

+1  Y   Q   Q   K   P   G   Q   P   P   K   L   L   I   Y   L
586  TAC CAA CAG AAA CCA GGA CAG CCA CCC AAA CTC CTC ATC TAT CTT
    ATG GTT GTC TTT GGT CCT GTC GGT GGG TTT GAG GAG TAG ATA GAA

+1  A   S   N   L   E   S   G   V   P   A   R   F   S   G   S
631  GCA TCC AAC CTA GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC AGT
    CGT AGG TTG GAT CTT AGA CCC CAG GGA CGG TCC AAG TCA CCG TCA

+1  G   S   G   T   D   F   T   L   N   I   H   P   V   E   E
676  GGG TCT GGG ACA GAC TTC ACC CTC AAC ATC CAT CCT GTG GAG GAG
    CCC AGA CCC TGT CTG AAG TGG GAG TTG TAG GTA GGA CAC CTC CTC

+1  E   D   A   A   T   Y   Y   C   Q   H   S   R   E   L   P
721  GAG GAT GCT GCA ACC TAT TAC TGT CAG CAC AGT AGG GAG CTT CCT
    CTC CTA CGA CGT TGG ATA ATG ACA GTC GTG TCA TCC CTC GAA GGA

+1  R   T   F   G   G   G   T   K   L   E   I   K   R   |   Spacer
766  CGG ACG TTC GGT GGA GGC ACC AAG CTG GAA ATC AAA CGG | GCG GCC
    GCC TGC AAG CCA CCT CCG TGG TTC GAC CTT TAG TTT GCC | CGC CGG

+1  A   |   Myc-tag   |   Spacer
811  GCA | GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AAT GGG | GCG GCA
    CGT | CTT GTT TTT GAG TAG AGT CTT CTC CTA GAC TTA CCC | CGC CGT

+1  H   H   H   H   H   H   *
856  CAT CAC CAT CAC CAT CAC TAA
    GTA GTG GTA GTG GTA GTG ATT

```

Fig. 35-2

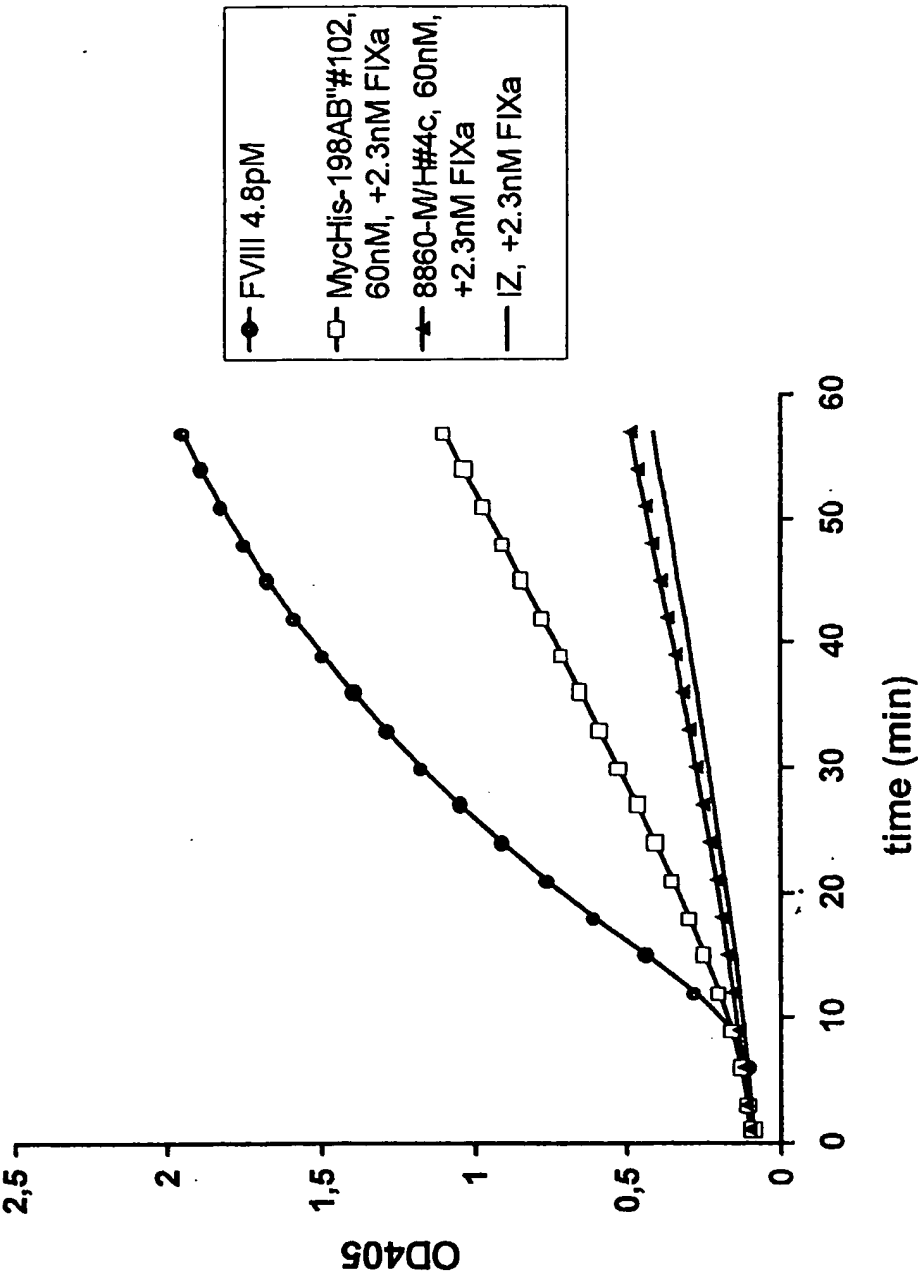


Fig. 36